Università degli Studi di Cagliari

DOTTORATO DI RICERCA IN SCIENZE MORFOLOGICHE

XXIII ciclo

TITOLO TESI

MACRO-MICRO NUTRIENTS INTERACTIONS: DIETARY FATS' INFLUENCE ON VITAMIN A METABOLISM

Settore/i scientifico disciplinari di afferenza

BIO 09

Presentata da:	Dott.ssa Elena Giordano
Coordinatore Dottorato	Prof.ssa Valeria Sogos
Relatore	Prof. Sebastiano Banni

Esame finale anno accademico 2009 – 2010

Table of contents

INTRODUCTION 1

1.The importance of fats in the diet 1

2. CONJUGATED LINOLEIC ACID 7

2.1 *CLA metabolism* *8*

2.2 *Biological effects and mechanisms of action* *9*

2.3 *Beneficial and detrimental biological health effects of CLA* *11*

2.4 *CLA dietary consumption* *15*

3 .VITAMIN A 17

3.1 *Definition and importance in the diet* *17*

3.2 *Dietary CLA perturbs vitamin A metabolism* *21*

4 .STUDY RATIONALE AND OBJECTIVES 26

5 .EXPERIMENTAL DESIGN 28

6 .MATERIALS AND METHODS 31

7 .RESULTS 35

7.1 Effect of an acute dietary CLA administration on vitamin A intestinal absorption *35*

7.2 Tissue distribution of recently ingested vitamin A *43*

7.3 Effect of a chronic dietary CLA intake on circulating retinol and RBP levels *52*

7.4 Effect of an acute dietary CLA intake on vitamin A tissue distribution *53*

7.4 Effect of an acute dietary intake of Sunflower oil, Flaxseed oil and Olive oil on vitamin A intestinal absorption *59*

8 .DISCUSSION 65

9 .CONCLUSIONS AND FUTURE PERSPECTIVES 75

BIBLIOGRAPHY 77

ACKNOWLEDGMENTS 86

Introduction

1. The importance of fat in the diet

Diet provides the necessary macronutrients as proteins (or their building blocks, aminoacids), carbohydrates and lipids, needed for energy production, tissue anabolism, precursors of bioactive molecules, which in concert contribute to body composition homeostasis. In addition, numerous micronutrients are required by living organisms throughout life in smaller quantities to orchestrate a whole range of physiological functions, namely as essential role as protein cofactors in metabolism and as regulators of metabolic functions as cell or tissue growth and differentiation and antioxidants. Micronutrients are as well provided with the diet. They include macrominerals (e.g. Calcium, Magnesium, Phosphorus, Potassium), micro or trace minerals (e.g. Iron, Manganese, Fluoride, Zinc, Iodine), organic acids (e.g. acetic acid, citric acid, lactic acid) and a whole variety of fat-soluble and water-soluble vitamins, including vitamin A (Ross 2010)

Fats, waxes, sterols and a wide array of neutral (mono, di and triacylclycerols) and polar lipids (e.g. phospholipids, sphingolipids) belong to the broad group of naturally occuring organic materials generally called lipids. Lipids are those constituents of plants or animals that are insoluble in water (hydrophobic), but soluble in other organic solvents. Some lipids are amphiphilic, i.e. they possess polar domains that interact both with lipophylic and aqueous environments. Therefore they are essential to form cellular structures as vesicles, liposomes or membranes. Lipids are also concentrated sources of carbon and energy that are used to fuel organism activities and growth. All living organisms need a certain amount of dietary fat for normal body functioning. When fats are digested, emulsified, and absorbed, they facilitate the intestinal absorption and transport of fat-soluble or lipophylic vitamins A, D, E, and K. Fat also surrounds vital organs as the heart, kidneys, and mammary gland, either to quickly provide fat soluble molecules and to cushion and protect them.

The digestion of lipids begins in the oral cavity through exposure to lingual lipases, which are secreted by exocrine glands in the tongue to begin the process of digesting triglycerides. Digestion continues in the duodenum, which is the major site for the emulsification of dietary fat and fat-soluble vitamins. Crude lipids enter the duodenum and are mixed up with bile acids that play their first critical role in lipid assimilation by promoting emulsification. As derivatives of cholesterol, bile acids have both hydrophilic and hydrophobic domains (i.e. they are

amphipathic). On exposure to a large aggregate of triglyceride, the hydrophobic portions of bile acids intercalate into the lipid, with the hydrophilic domains remaining at the surface. Such coating with bile acids aids in breakdown of large aggregates or droplets into smaller and smaller droplets (Figure 1).

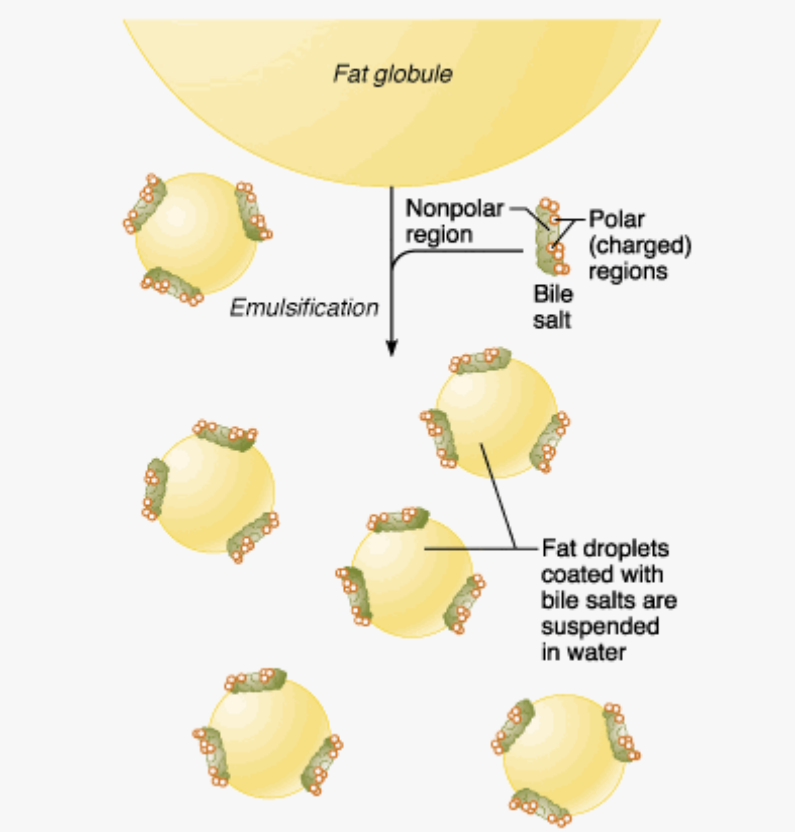

Figure 1. Emulsification of lipids by bile salts.

Hydrolysis of triglyceride into monoglyceride and free fatty acids is accomplished predominantly by pancreatic lipase. The activity of this enzyme is to clip the fatty acids at positions 1 and 3 of the triglyceride, leaving two free fatty acids and a 2-monoglyceride. Lipase is a water-soluble enzyme which is present in high quantities within the small intestine shortly after a meal, but can act only on the surface of triglyceride droplets. As monoglycerides and fatty acids are liberated through the action of lipase, they retain their association with bile acids and complex with other lipids to form structures called micelles. Micelles are essentially small aggregates (4-8 nm in diameter) of mixed lipids and bile acids

suspended within the ingesta. As the ingesta is mixed, micelles bump into the brush border of small intestinal enterocytes, and the lipids, including monoglyceride and fatty acids, are taken up into the epithelial cells. The major products of lipid digestion, free fatty acids (FFAs) and 2-monoglycerides (2-MAGs). 2-MAG is the predominant form in which MAG is absorbed from the small intestine.

Further hydrolysis of 1- or 2-MAG by pancreatic lipase results in the formation of glycerol and FFAs (Hofmann et al. 1963); cholesterol esterase can also hydrolyze the acyl group at the *sn*-2 position to form glycerol and FFAs (Lombardo et al. 1980). A considerable fraction of the fatty acids also enter the enterocyte via a specific fatty acid transporter protein in the membrane. Once inside the enterocyte, fatty acids and monoglyceride are transported into the endoplasmic reticulum, where they are used to synthesize triglyceride. Beginning in the endoplasmic reticulum and continuing in the Golgi, triglyceride is packaged with cholesterol, lipoproteins and other lipids into particles called chylomicrons (Hussain 2000).

Chylomicrons (Figure 2) are large lipoprotein particles that consist of triglycerides (85-92%), phospholipids (6-12%), cholesterol (1-3%) and proteins (1-2%). They transport exogenous lipids to liver, adipose, cardiac, and skeletal muscle tissue, where their triglyceride components are unloaded by the activity of lipoprotein lipase. As a consequence, chylomicron remnants are left over and are taken up by the liver.

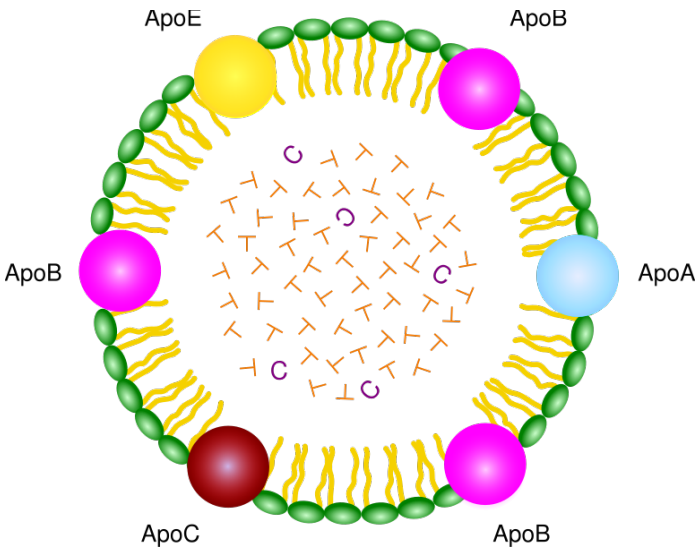

Figure 2. Chylomicron structure. ApoA, ApoB, ApoC, ApoE (apolipoproteins); T (triacylglycerol); C (cholesterol); green (phospholipids)

There are three stages in the chylomicron's "life cycle":

- Nascent chylomicron
- Mature chylomicron
- Chylomicron remnant

Nascent chylomicrons:

These particles are created by the absorptive cells of the small intestine, known as enterocytes. They are relatively large, having a diameter of 75 to 1,200 nm. These nascent chylomicrons are released by exocytosis from enterocytes into lacteals, lymphatic vessels originating in the villi of the small intestine, and are then secreted into the bloodstream at the thoracic duct's connection with the left subclavian vein. They are primarily composed of triglycerides (85%) and contain some cholesterol and cholesteryl esters. The main apolipoprotein component is apolipoprotein B-48 (APOB48). They also contain newly formed retinyl esters that mix with other neutral lipids during assembly of the lipid core of the nacent chylomicrons. The transport of retinyl ester by chylomicrons is important for the delivery of vitamin A to tissues that metabolize these triglyceride-rich lipoproteins

Mature chylomicron:

While circulating in lymph and blood, chylomicrons exchange components with high-density lipoproteins (HDL). The HDL donates apolipoprotein C-II (APOC2) and apolipoprotein E (APOE) to the nascent chylomicron and thus converts it to a mature chylomicron. APOC2 is the cofactor for lipoprotein lipase (LPL) activity.

Chylomicron remnant:

Once triglyceride stores are distributed, the chylomicron returns APOC2 to the HDL (but keeps APOE), and, thus, becomes a chylomicron remnant, now only 30–50 nm. APOB48 and APOE are important to identify the chylomicron remnant in the liver for endocytosis and breakdown.

Fatty acids are carboxylic acids composed of a long unbranched carbon chain, which can be either saturated or unsaturated depending whether it solely contain single bonds (saturated, SFA) or instead one (monounsaturated, MUFA) or multiple double bonds (polyunsaturated, PUFA) between consecutive carbon atoms along the chain, respectively (Fig. 1). Unsaturated fatty acids are usually in *cis* conformation since desaturases insert double bonds in cis position, however, *trans* isomers may also be formed during industrial or bio hydrogenations. According to the first double bond position starting from the methyl group, fatty acids are classified into omega omega (ω or n) classes (Fig.3). Geometrical and positional isomers of the double bonds modify the reactivity of the fatty acid and are fundamental to confer them distinct biological properties.

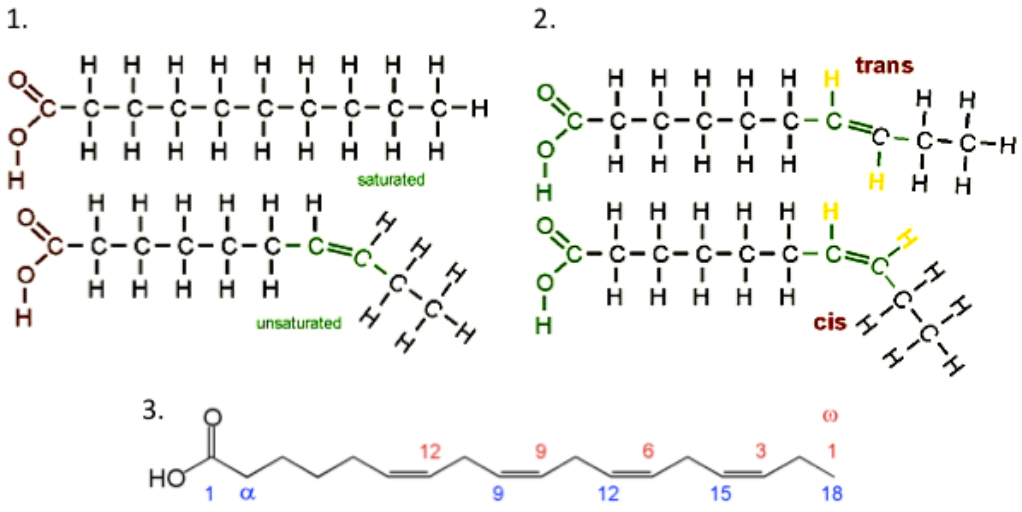

Figure 3. General structure and nomenclature of fatty acids. **1.** Saturated fatty acids possess only single bonds between adjacent carbon atoms, whereas unsaturated fatty acids posses one or several double bonds being referred as monounsaturated (MUFA) or polyunsaturated (PUFA) respectively. **2.** Fatty acids can display *trans* or *cis* configuration. A *cis* configuration means that adjacent hydrogen atoms are on the same side of the double bond. The rigidity of the double bond freezes its conformation and, in the case of the *cis* isomer, causes the chain to bend and restricts the conformational freedom of the fatty acid. The more double bonds the chain has in the *cis* configuration, the less flexibility it has. *Cis* bonds limit the ability of fatty acids to be closely packed, and therefore could reduce the melting temperature of the membrane or fat. Examples of *cis* fatty acid are oleic, linoleic acid. *Trans* configuration, by contrast, means that the next two hydrogen atoms are bound to opposite sides of the double bond. As a result, they do not cause the chain to bend much,

and their shape is similar to straight saturated fatty acids and therefore increases the melting point and rigidity of membranes. **3.** Nomenclature: In the n or ω a double bond is located on the x^{th} carbon–carbon bond, counting from the terminal methyl carbon toward the carbonyl carbon. Fatty acid carbon counting can be also initiated from the carbonyl terminal (which is carbon number 1).

In terms of human health, a recent meta-analysis has put more doubts on the overall relationship between saturated fatty acids and coronary heart disease7. In the work of Siri-Tarino and co-workers (Siri-Tarino et al. 2010), which included 21 human studies, the authors found that there is no substantial evidence for concluding that dietary saturated fat is associated with an increased risk of heart disease. Insufficient evidence exists to judge the effect on CHD risk of replacing SFAs with MUFAs. No clear association between SFA intake relative to refined carbohydrates and the risk of insulin resistance and diabetes has been shown. The effect of diet on a single biomarker is insufficient evidence to assess CHD risk. The combination of multiple biomarkers and the use of clinical endpointscould help substantiate the effects on CHD. Furthermore, the effect of particular foods on CHD cannot be predicted solely by their content of total SFAs because individual SFAs may have different cardiovascular effects and major SFA food sources contain other constituents that could influence CHD risk (Astrup et al. 2011) Research is needed to clarify the role of SFAs compared with specific forms of carbohydrates in CHD risk and to compare specific foods with appropriate alternatives.

2. Conjugated Linoleic Acid

The term conjugated linoleic acid, also referred as CLA, refers to a mixture of positional and geometric isomers of linoleic acid (18 carbon fatty acid), characterized by conjugated double bonds that are not separated by a methylene group as in linoleic acid (Fig. 4). These double bonds are usually located at positions 8 and 10, 9 and 11, 10 and 12, 11 and 13, and can occur both in *cis* or *trans* configuration. CLA isomers are found mainly in meat and dairy products from cows and sheep.(Churruca et al. 2009)

The most abundant isomer is c9,t11, which represents up to 80% of total CLA in food.

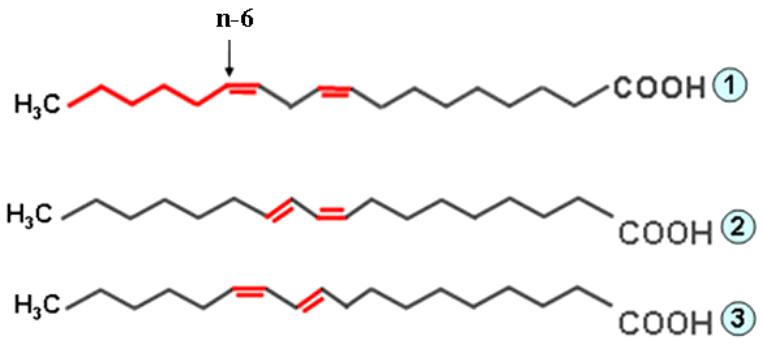

Figure 4. Structure of linoleic acid and its major CLA derivatives. **1.** Linoleic acid (typical n-6 PUFA); **2.** *c9,t11*-octadecadienoic acid (9-CLA, in fact an n-7 fatty acid); **3.** *t10,c12* octadecadienoic acid (10-CLA).

CLA *c9,t11* is synthesized through the activity of the bacteria *Butyrivibrio fibrisolvens* (Class Clostridia), that is commonly present in the rumen of ruminant animals (e.g. cows, sheep, deer, goats) and that is able to isomerize linoleic acid (*LA cis*-9, 12) producing CLA *cis*-9, *trans*-11 (Fukuda et al. 2006). CLA *cis*-9, *trans*-11 is then further reduced to *trans*-11 octadecanoic acid (vaccenic acid). After its absorption, vaccenic acid can be stored or be converted into *cis*-9, *trans*-11 by incorporating a *cis* double bond in position 9, due to the activity of Δ9 desaturase enzyme that is mainly active in ruminant mammary gland.

It must be pointed out that there are other bacteria that have the ability to synthesize CLA, such as those from the genus *Propionibacter*. Therefore, the nature of the microbial population in the rumen, and other factors such as the type of food provided to the animals, affect the total amount and proportion of the CLA isomers found in tissues and milk. The amounts of CLA are higher in meat and milk from cows fed with fresh green forages, or which are fed with supplements of sunflower or soybean oils, than in animals fed with grain or forage. CLA can also be synthesized by partial hydrogenation processes used in the food industry for the development of shortenings such as margarines. In these products the amount of CLA depends on the type of hydrogenation (Jung et l. 1999).

2.1. CLA metabolism

The metabolism of CLA appears to be similar in rodents and humans. CLA isomers are metabolized through different pathways. In the liver and mammary glands of rats, as well as in the adipose tissue of humans, CLA metabolites which maintain the conjugated diene (CD) chemical structure, such as conjugated octadecetrienoic acid (CD 18:3), conjugated eicosatrienoic acid (CD 20:3), and conjugated eicosatetraenoic acid (CD 20:4) have been detected. These metabolites are formed by CLA isomer elongation and desaturation processes due to the action of Δ6 and Δ5 desaturases and an elongase. These conjugated dienes are different depending on their double bond configuration (*cis,trans*, *trans,cis*, or *trans,trans*) (Banni 2002).(Sebedio et al. 1997) Moreover, the similarity of the backbone structure of CLA with oleic acid results in its preferential incorporation in neutral lipids, and therefore in its preferential accumulation in tissues with an elevated content of neutral lipids, such as mammary gland and adipose (Sebedio et al. 1997). In contrast, linoleic acid and its metabolites are predominantly found in phospholipids. A metabolic competition between CLA and linoleic acid has been shown. Finally, CLA *c9,t11* and CLA *t10,c12* can be efficiently partially -oxidized probably in peroxisomes both *in vivo* and *in vitro* (Banni et al. 2004).

2.2. Biological effects and mechanisms of action of CLA

CLA was initially discovered accidentally when Pariza and Hargraves (Pariza et al. 1985) were investigating the carcinogenic properties of ground beef, and contrary to their expectations, that fatty acid exhibited anticarcinogenic rather than procarcinogenic properties. The anticarcinogen factor was then purified and identified as four isomers of linoleic acid with conjugated diene unsaturatio . Later it was reported that CLA suppressed tumor development in a range of animal models and inhibited growth in many cancer cell lines (Churruca et al. 2009)

There are twenty eight possible isomers of CLA existing in nature, but the major isomer in natural foods is the CLA *c9,t11* accounting for more the 90% CLA intake in the diet (Fritsche et al. 1999). Health benefits have been attributed mainly to the *c9,t11* and/or *t10,c12* isomers. Intake of these two isomers has been reported to induce several beneficial effects in health related disorders, using animal models and cell cultures derived from humans and animals (Bhattacharya et al. 2006).Thus CLA has been shown to have antiadipogenic, anticarcinogenic, antiatherogenic, antidiabetogenic and antinflammatory properties (Kelly 2001).

Many studies were performed using a mixture of CLA *c9,t11* and CLA *t10,c12*, usually at a ratio of approximately 1:1, but with the advent of new technologies, enriched or purified *c9,t11* and *t10,c12* CLA preparations have become commercially available, leading to studies examining the effects of each individual isomers. Recent evidences suggest that both isomers may have similar but also opposite biological activities, however the reasons for such distinct actions have not been fully elucidated yet (Benjamin et al. 2009). The CLA action at molecular level lies predominantly on the classical CLA-mediated activation of peroxisome proliferator activated receptors (PPARs) and subsequent "switching on and/or off" of the target genes to elicit multiple biochemical pathways (Benjamin et al. 2009). As shown in Figure 5, during gene regulation, the well-characterized PPARs (α, β or γ subtypes) bind to the peroxisome proliferator responsive element (PPRE) on the nuclear DNA as heterodimers with binding protein (SREBP) (Sampath et al. 2005).

Modulatory effects of CLAs juxtaposed to PPAR-mediated gene expression to effect novel molecular signalling pathways, which are largely mediated though leptins (Perez-Matute et al.

2005), adiponectin, eicosanoids, vitamins, immunoglobulins , and thus altering membrane protein characteristics [33] are also being elucidated. However, objective evidences for these pathways are still inconclusive.

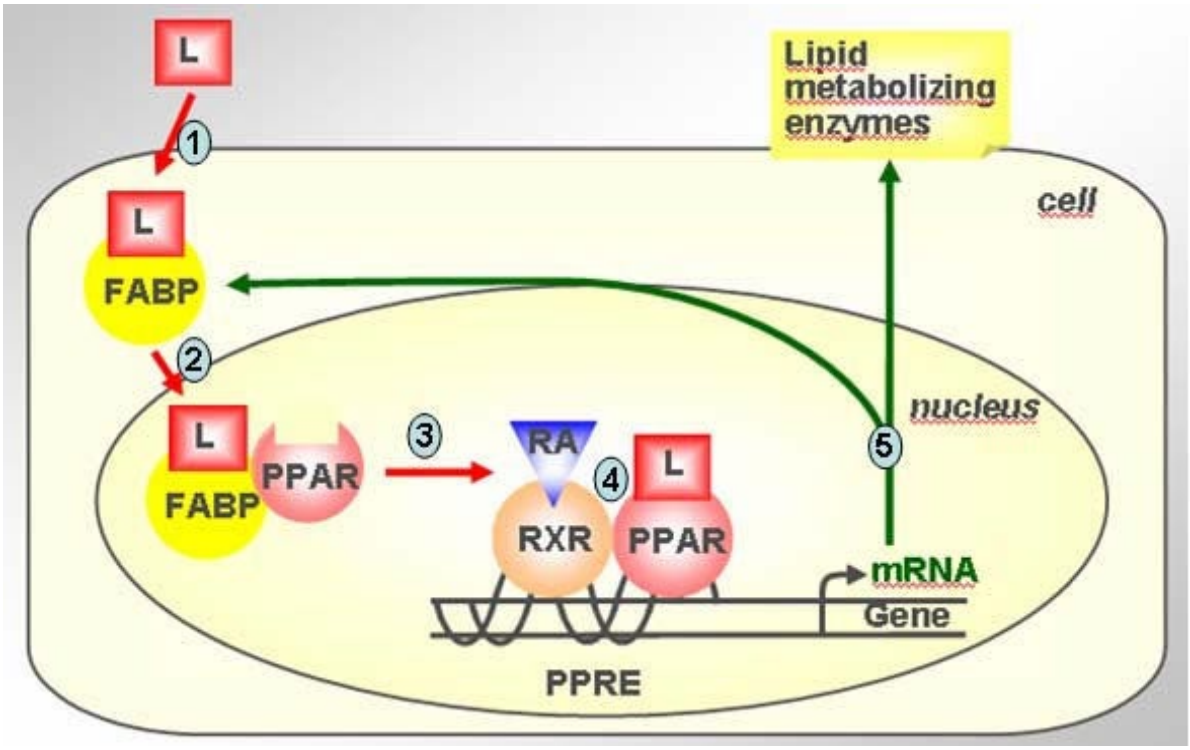

Figure 5. Proposed CLA-mediated signal transduction. *The dietary lipid nutrient L (L = here CLA) crossing the cell membrane with the help of specific membrane-bound fatty acid transporters and binds to tissue-specific fatty acid binding protein (FABP) in the cytosol; 2. The L/FABP complex enters in to the nucleoplasm, where L is transferred to the specific peroxisome proliferators activated receptor (PPAR) subtype; 3. The L/PPAR complex heterodimerises with retinoic acid (RA)/retinoic acid receptor (RXR) subtype; 4. This heterodimer binds to the peroxisome proliferator responsive element (PPRE) on the target gene; and 5. Specific gene expression occurs, whose products act intra- or extracellularly to elicit a host of various biological functions. (Benjamin et al. 2009)*

2.3. Beneficial and detrimental biological health effects of CLA

In this context, it is appropriate to have an overview into some of health benefits attributed to CLA, including anti-obesity, anti-carcinogenic, anti-atherogenic, immunomodulatory effects, and also its possible negative effects like fatty liver and spleen, induction of colon carcinogenesis and hyperproinsulinaemia (Bhattacharya et al. 2006)

Park et al. (Park et al. 1997) were the first authors to demonstrate that CLA modulated body composition in rodents. In their study, male and female mice given a 0.5% CLA mixture had 57% and 60% lower body fat mass (BFM), respectively, than controls. Other researchers have subsequently demonstrated that *t10,c12* isomer is primarily involved in reduction of fat mass; when hamsters were fed with a hypercholesterolemic diet containing 1% CLA, 0.2% *c9,t11* CLA or 1% LA, CLA isomer mix-fed animals had the lowest weight gain. Yet, another study showed that intake of *t10,c12* isomer enriched diet decreases body fat significantly compared to diet enriched in c9t11 isomer.(Clément et al. 2002)

Currently, the experimental trend is to use a single isomer (*mostly cis 9, trans11 or trans10, cis 12* CLA) with purity above 90%. Apart from the specific anti-obesity and hypocholesterolaemic effects of *t10,c12* CLA [40], most of the rodent (rat and mice) studies suggest that a CLA mixture *(c9,t11* + *t10,c12* CLA) could be more beneficial for the management of insulin resistance (Benjamin et al. 2009).

Studies investigating CLA's effects on BFM (body fat percentage) reduction in humans have produced less consistent results. For example, moderately overweight humans with an average weight of 72.5 kg supplemented with a CLA mixture (3.76 g/day for 14 weeks) experienced no decrease in body weight, BMI or BFM. In contrast, C57BL/6 mice supplemented with 1.5% (wt/wt) CLA mixture for 4 weeks weighed significantly less and had reduced adiposity compared to controls. However, while subjects in the human study received approximately 0.05 g/kg body weight CLA, the mice received 1.07 g/kg body

weight CLA, which was 20 times the human dose based on body weight. Supplementing humans with higher doses of CLA would address this dosing issue (Kennedy et al. 2010). Moreover, most animal studies have been in young growing mice or rats, whereas studies in humans were mostly in mature volunteers. As noted earlier, the effects of CLA on body

composition in mature animals have not been as dramatic as those seen in young animals. (Bhattacharya et al. 2006)

CLA has also been reported to have antiatherosclerotic effects, mainly through its ability to alter hepatic lipid and lipoprotein metabolism (Churruca et al. 2009)There are indications that the effects of CLA on blood lipids may be different depending on the CLA isomer used. In addition, although these effects are relatively dramatic in animal studies, they are not consistently supported by human studies. Therefore, a general consensus has not been reached.

Concerning animal models, it must be pointed out that it is difficult to make comparisons among studies because of the existence of important differences in experimental conditions: animal species, CLA amount and isomeric composition, treatment duration, basal serum lipids in animals, diet composition,etc. In these terms although the effects of CLA on blood lipids have been studied in several animal models such as hamsters, rats, mice and rabbits, the reported results show either increases, decreases or no changes in plasma triglycerides, total cholesterol, HDL or non-HDL cholesterol. Moreover, to compare these studies it should be pointed out that there are important differences in animal basal lipoprotein profiles. This initial lipoprotein profile depends on the fatty acid profile of diets used in each experiment. The mentioned beneficial changes in blood lipids have been observed by using either a CLA mixture or the *t10,c12* isomer, suggesting that these effects are attributable to this isomer. However, Valeille et al. (Martin et al. 2002)published an article in which it was demonstrated that the active isomer of CLA was *c9,t11*. Although the published results concerning the effects of CLA on serum lipids are conflicting, the balance of evidence suggests that, under some experimental conditions, CLA develops a less atherogenic lipoprotein profile. Further studies should be carried out to define the best animal model (species, metabolic status) to use in these studies. (Bhattacharya et al. 2006).

CLA has also been shown to prevent formation of atherosclerotic lesions in rabbits, hamsters and in atherosclerosis-prone ApoE knockout mice fed a 1% cholesterol diet. One percent CLA has been shown to cause 30% regression of established atherosclerosis in rabbits that had been rendered atherosclerotic through 0.1–0.2% cholesterol diet for 90 days. As little as 0.05% CLA was shown to reduce severity of atherosclerotic lesions in rabbits; this increased inversely with CLA content in diet, which is a particularly interesting observation considering the low CLA content in the diet, which may be attainable in human diets. Numerous other

studies in hamsters seem to suggest that CLA or the individual isomers may be more effective against atherosclerosis when diet is high in saturated fat content.

The underlying mechanisms involved in the antiatherosclerotic and lipid-lowering effects of CLA or individual isomers have not been adequately addressed both *in vitro* and *in vivo* studies. Some of the proposed mechanisms include their role on peroxisome proliferator-activated receptors (PPARs), sterol regulatory element-binding proteins (SREBPs) and SCD. Peroxisome proliferator-activated receptors are ligand-activated nuclear receptors regulating the expression of genes that control lipid and glucose homeostasis, thus modulating the major metabolic disorders predisposing to atherosclerosis. Moreover, PPARs exert additional anti-inflammatory and lipid-modulating effects in the arterial wall, therefore being interesting molecular targets for the treatment of atherosclerosis. PPAR α regulates the expression of genes involved in fatty acid oxidation and energy homeostasis. Natural ligands of the PPARα include fatty acids and their derivatives (eicosanoids, 8S-HETE) and leukotriene B4 .

PPAR γ is expressed primarily in adipose tissue and induces expression of genes that promote lipid storage including lipoprotein lipase that is critical in the removal of TG-rich lipoproteins. Studies with pure isomers suggest that *c9,t11* isomer is more effective than *t10,c12* isomer in modulating key modulators of lipid metabolism. Although both *c9,t11* and *t10,c12* isomers are ligands for PPAR-activated receptor PPARα, results suggest that *c9,t11* isomer is the more potent activator of the two.

However, the recent finding that CLA-fed PPARα null mice have lower plasma TG levels suggests that lipid-lowering effects of CLA may be independent of PPARα .

Sterol regulatory element-binding protein 1 isoforms regulate fatty acid and TG synthesis. Studies suggest that liver SREBP-1c expression is dependent on the nuclear hormone receptor liver X receptor (LXR). *C9,t11* isomer was shown to down-regulate mRNA expression of LXR-a and SREBP-1c, whereas the *t10,c12* isomer had no effect. The results suggested that *c9,t11* isomer positively influences lipid metabolism by reduced synthesis and cleavage of hepatic SREBP-1, which in turn is regulated by hepatic LXRa expression. Sterol regulatory element-binding protein 1c enhances the transcription of the genes required for fatty acid synthesis and fatty acid elongation including FAS and SCD. Stearoyl-CoA desaturase gene expression is highly regulated and may be influenced by dietary lipids, hormones, peroxisomal proliferators, etc. The SCD1-deficient animals produce low levels of

VLDL, suggesting that the rate of VLDL production might itself be influenced by SCD1 activity. Both isomers have been shown to inhibit SCD activity in breast tumor cell lines but in HepG2 cells and 3T3-L1 cells, *t10,c12* isomer showed lower SCD activity and expression compared to the c9t11 isomer. Thus, inhibition of SCD1 activity could be one of the mechanisms involved in the lipid-lowering effect of CLA .

Hypertension is also a common pathological state associated with an increased risk of cardiovascular diseases. Nagao et al. have consistently shown that CLA (50:50) or the *t10,c12* isomer, but not *c9,t11* isomer, decreases blood pressure and hypertension in various rat models prone to develop obesity, diabetes and obesity together, or hypertension (Bhattacharya et al. 2006).

The possible beneficial effects of CLA supplementation in decreasing body fat mass have received a great deal of attention, but potential adverse effects of CLA on insulin sensitivity are a matter of concern. Conflicting results have been published concerning this issue. Thus, whereas in Zucker diabetic fatty rats (ZDF) and fa/fa Zuker rats, CLA improves glucose tolerance [37,38], it induces insulin resistance in mice [39,40]. There is also conflicting information as to which CLA isomer(s) could be responsible for glucose tolerance changes. In rodents, it has been demonstrated that a CLA mixture (50:50 product) and the *t10,c12* isomer, but not *c9,t11*, improves glucose tolerance in fa/fa Zucker rats and ZDF rats [38,41].

In AKR/J mice (a strain susceptible to dietary obesity), adding CLA as one percent of dietary calories resulted in a nearly two-fold increase in plasma insulin levels. In these mice there was also a trend toward higher blood glucose levels. The combination of both higher circulating insulin and glucose levels suggests impairment in the ability of insulin to dispose of glucose in tissues, a finding consistent with the development of an insulin resistant state. Even though these mice had a reduction in adipose tissue weight, the development of an insulin resistant state would not be construed as a beneficial metabolic change (Kelly 2001).

In obese men showing signs of metabolic syndrome the *t10,c12* isomer was reported to markedly decrease insulin sensitivity [42]. Later on, this research group conducted a double-blind placebo-controlled study in abdominally obese men treated with *c9,t11* CLA. They observed that this isomer also decreased insulin sensitivity compared with the placebo. This report was the first to suggest some detrimental effects of *c9,t11* CLA on human health. As both CLA isomers appear to decrease insulin sensitivity it is, however, quite surprising that such an effect was not observed when a 50:50 mixture of CLA isomers was given as a

supplement [43]. In another study, Tricon et al. [44] suggested that *t10,c12* CLA increased fasting blood glucose concentration relative to the *c9,t11* isomer in healthy men, although this effect was found to be insufficient to modify the extent of insulin resistance or insulin sensitivity. One possible explanation of this apparent discrepancy, is that the insulin resistance is a transient effect due to a diversion in the utilization of energy substrate favoring fatty acid beta oxidation and sparing glucose which triggers insulin resistance particularly in subjects with low metabolic active (lean) mass and high visceral fat mass.

2.4. CLA dietary consumption

Overall, there are considerable variations between studies, and the beneficial effects observed in some animal models have not been fully reproduced in humans. Dosage, duration of the studies, metabolic state of the animals and the strain used, differences in composition and sources of CLA, as well as age, sex and health status of the human subjects may be responsible for the different results. More investigations are needed to define the mechanisms of action of CLA and to establish whether CLA could effectively and safely prevent diseases in humans. Nevertheless, consumption of large doses of CLA in the form of dietary supplements has become a very common practice in recent years, Although the daily average intake of CLA from natural products does not exceed 500mg {Brownbill, 2005 #835}, CLA intake can dramatically increase due to ingestion of food that is being fortified with CLA and/or of large doses of CLA supplements. Interestingly, a daily CLA intake of 3g has been recently recommended and considered safe by the European Food Safety Authority (EFSA) {Authority, 2010 #836} and it received the GRAS approval by USDA.

Hence, caution should be used in recommending CLA to prevent human diseases until its health benefits are fully proven and the mechanisms of CLA actions entirely understood. In this respect, it is very intriguing that dietary CLA perturbs retinoid metabolism as previously shown from Banni and colleagues. They noted that the addition of a mixture of CLA isomers (50% CLA *cis 9, trans11* and 50% CLA *t10,c12*) to the diet of female rats for four weeks unexpectedly resulted in increased concentrations of serum retinol and elevated levels of retinol and retinyl ester in liver and mammary tissue. And more recent published data from

Quadro's laboratory (Ortiz et al. 2009) demonstrated for the first time that CLA *cis 9, trans11* and *t10,c12* have specific effects on vitamin A metabolism.

Vitamin A

3.1 Definition and importance in the diet

Vitamin A is a lipo-soluble vitamin that cannot be synthesized endogenously by humans and thus, it is an essential nutrient. Vitamin A or derived metabolites (retinyl esters, retinol, retinoic acid, also known as retinoids) play an important role in a number of physiological processes including vision (color vision, low-light vision), cell differentiation, reproduction and immunity and regulates the transcription of a number of genes that are crucial for many important biological functions (Blomhoff 1994) (Figure 6).

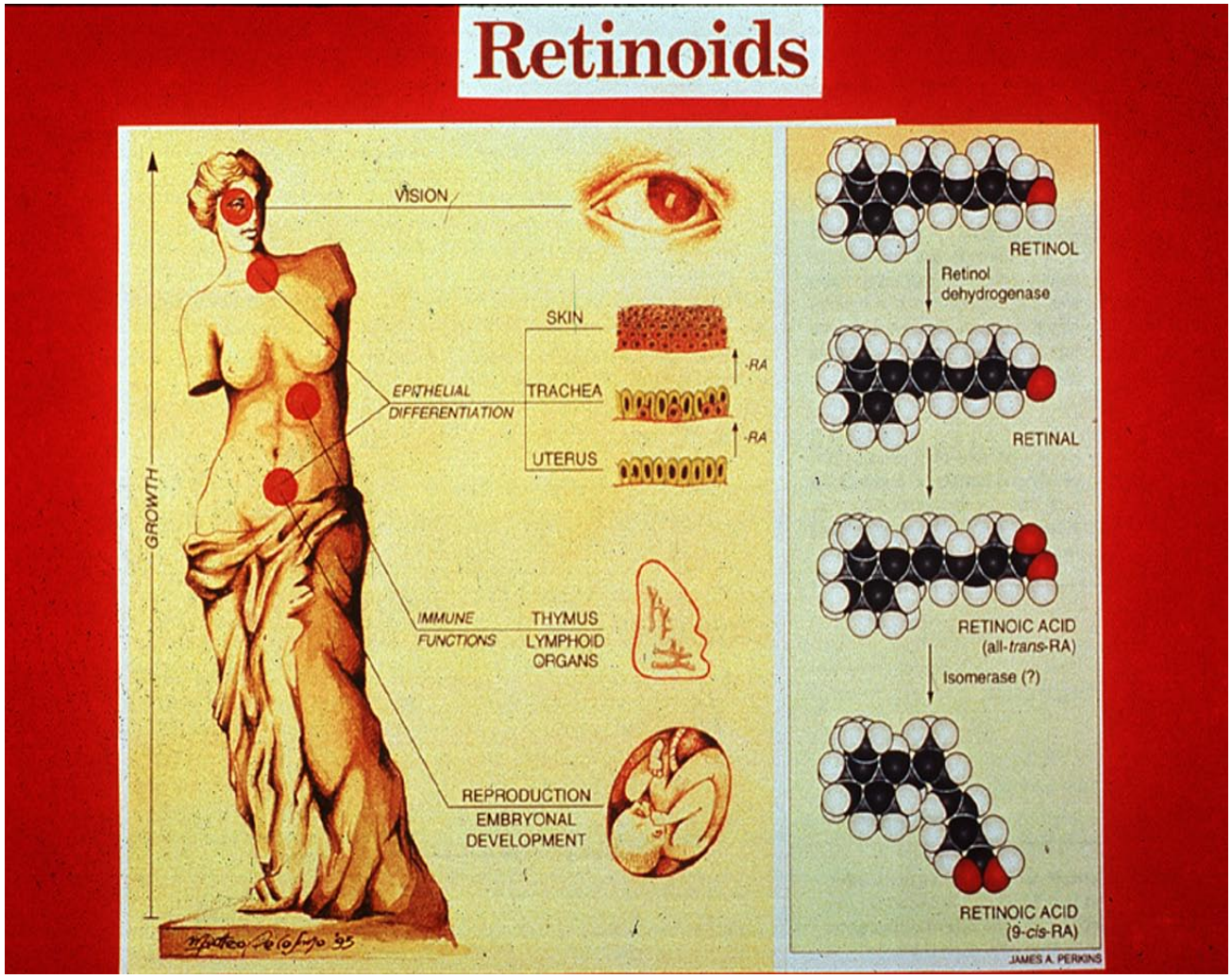

Figure 6. Biological function of vitamin A and its derivatives

Adequate levels of retinoids in serum and tissues are essential to maintain the health of the body. Retinoids homeostasis is achieved through a series of complex mechanisms that regulate absorption, storage, transport and metabolism of this nutrient. Mammals obtain all vitamin A and its derivatives (retinyl esters, retinol and very small amounts of retinoic acid) directly from animal products or as β-carotene from vegetables and fruits. (Blomhoff 1994).
As shown in figure 7, within the intestinal mucosa all retinol (ROH), regardless of its dietary origin, is enzymatically re-esterified with long-chain fatty acids primarily by the action of the enzyme lecithin: retinol acyltransferase (LRAT), which is widely expressed in tissues (Batten et al. 2004; Liu et al. 2005; O'Byrne et al. 2005). Together with other dietary lipids, the newly synthesized retinyl esters (RE) are packaged into chylomicrons that are large lipoproteins particles composed mainly of triglycerides but also phospholipids, cholesterol and proteins. Chylomicrons are secreted into the lymphatic system (Vogel et al. 1999) and once in the general circulation the lipoprotein lipase (LPL) that is bound to the luminal surface of the vascular endothelium, catalyzes the lipolysis of the triglycerides giving rise to free fatty acids and chylomicron remnants (Olivecrona et al. 1993; Goldberg 1996). After chylomicron remnants acquire apolipoprotein E, either in the plasma or in the space of Disse (the space between sinusoidal endothelium and hepatocytes), approximately 75% of chylomicron remnants retinoid, is cleared by the liver that is the major site of vitamin A storage and metabolism (Blaner et al. 1994; Cooper 1997). The remaining 25% is cleared by extrahepatic tissues (Goodman et al. 1965)

ROH absorption is impaired in essential fatty acid-deficient mice {Werner, 2002 #817}. Furthermore, Pluronic L-81, a hydrophobic surfactant that inhibits the secretion of chylomicrons, has been shown to block intestinal secretion of RE both *in vitro* and *in vivo* {Lissoos, 1995 #818; Schweigert, 2002 #830}. Also, patient with abetalipoproteinemia or hypobetalipoproteinemia, diseases due to a genetic absence of microsomal triglyceride transfer protein (MTP) that leads to impaired production of chylomicrons and VLDL, have impaired dietary vitamin A uptake {Chowers, 2001 #819}. These and other evidences strongly suggest that the rate-limiting step for the intestinal absorption of vitamin A is the formation and secretion of chylomicrons. This could be the reason why dietary fat is a major determinant of vitamin A absorption {Li, 2003 #820; Harrison, 2005 #689}. A significant

amount of ROH is also secreted probably as free ROH into the portal circulation where it would be absorbed on apo-RBP. However very little is known about the physiological significance and the regulation of intestinal ROH secretion by this pathway {Iqbal, 2009 #821; Harrison, 2005 #689}. It could represents an essential back-up mechanism for the homeostasis of vitamin A under certain conditions, such as impaired secretion of chylomicrons, for example (Harrison 2005).

Once taken up by the hepatocytes, RE are hydrolyzed again into ROH to be transferred to stellate cells and then re-esterified by LRAT for storage. Alternatively, ROH can bind to its specific transport protein, retinol-binding protein (RBP), to be secreted into the bloodstream (Soprano et al. 1994; Quadro et al. 2003). RBP is a 21 kDa protein with a single binding site for one molecule of all-*trans*-ROH. It is mainly, but not exclusively, synthesized within the hepatocytes (Soprano et al. 1994; Quadro et al. 2003). RBP circulates in the blood as a 1:1 molar complex with another serum protein, transthyretin (TTR) (Monaco et al. 1995). The major function of RBP is to mobilize hepatic retinoid stores and deliver ROH to peripheral tissues (Quadro et al. 1999; Quadro et al. 2003). The peripheral uptake of vitamin A, is mediated by Stra6, which is a large membrane protein recently identified as the receptor for the circulating complex ROH-RBP (Kawaguchi et al. 2007). In the fasting circulation, ROH-RBP accounts for approximately 99% of all serum retinoids. Upon vitamin A intake the concentration of chylomicrons and chylomicron remnants retinoids can greatly exceed the concentration of plasma ROH. Blood levels of ROH-RBP in both humans and animals are maintained very constant, except in extreme cases of nutritional intake of vitamin A, protein, calories and zinc; or in response to hormonal factors, stress and in certain disease states (Soprano et al. 1994; Biesalski et al. 1999; Yang et al. 2005). Intriguingly, elevated serum RBP levels {Yang, 2005 #454} and/or an altered ratio of circulating ROH-RBP {Mills, 2008 #822; Erikstrup, 2009 #823}, have been associated with insulin resistance. The mechanisms that regulate the secretion of the complex ROH-RBP from the liver have not been fully elucidated yet.

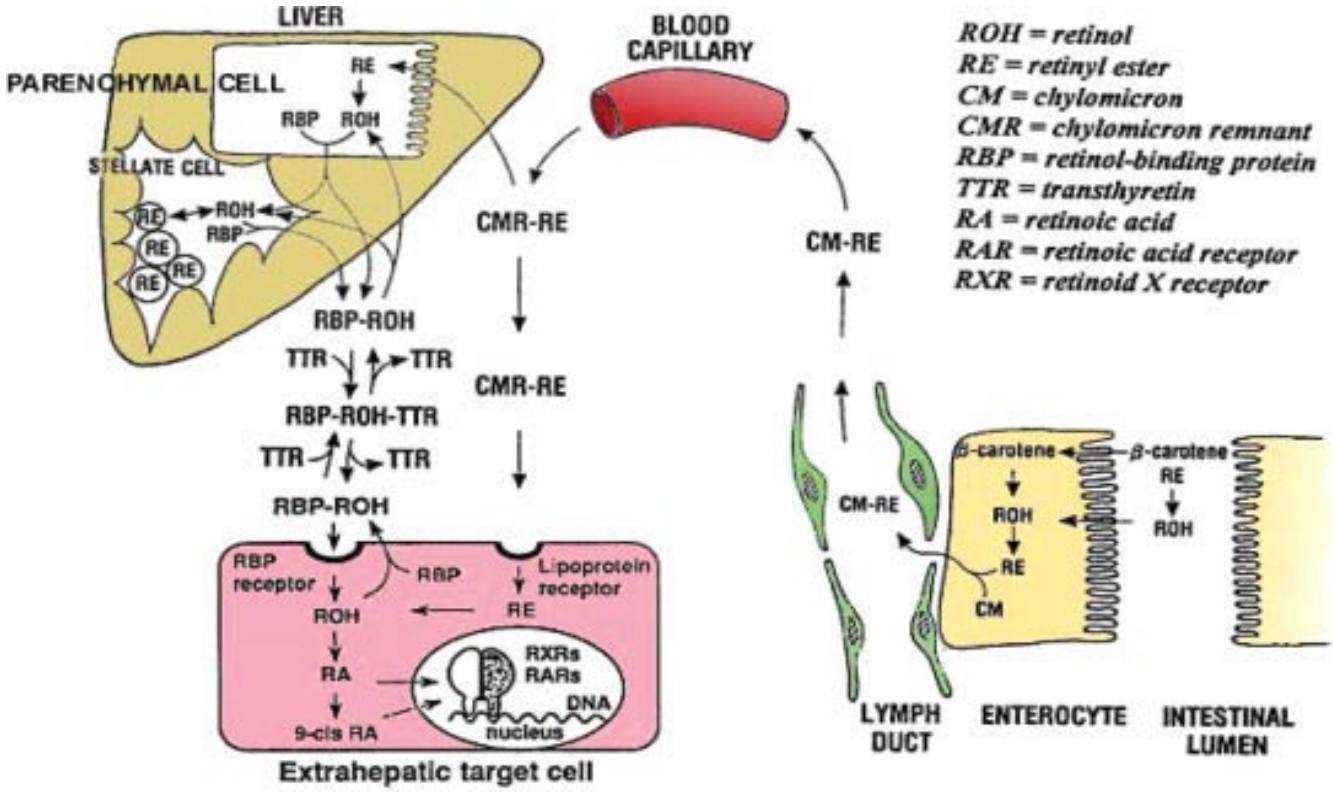

Figure 7. Major pathways for retinoid transport in the body. *All retinoids in animals are derived from the diet as preformed dietary vitamin A (RE, ROH, Retinoic acid) from animal products or from vegetables and fruit (β-carotene).In the intestinal lumen retinoids are incoroporated in micells that enter the enterocyte. In the intestine, dietary retinoids are incorporated in CM as RE (+ dietary lipids as TG, CE, PL). RE are taken by the hepatocites and hydrolized into RETINOL, which can be either tranferred to stellate cells and then re- esterified by LRAT for storage or can bind to its specific transport protein (RBP) to be secreted into the bloodstream (RBP circulates in the blood as a 1:1 molar complex with another serum protein, transthyretin (TTR) preventing retinol-RBP excretion by the kidney.*

3.2. Dietary CLA perturbs vitamin A metabolism

CLA has been shown to interfere with vitamin A metabolism. Specifically, with the addition of 0.5, 1.0, 1.5, or 2.0% CLA (in the form of a mixture of isomers) to the diet of rats for four weeks, the amount of retinol in liver and mammary tissue increased progressively, up to a maximum of a five-fold elevation (Banni et al. 1999). Hepatic retinyl esters also increased with a two-fold elevation and without a response to the amount of CLA given. Moreover, serum retinol levels also showed a statistically significant increase, albeit with no evident dose response effect (Banni et al. 1999). Although the mechanisms responsible for these effects remain unexplored to date, these findings suggest that the actions of CLA could be related, at least in part, to its ability to interfere with vitamin A metabolism.

Recently published data (Ortiz et al. 2009) have shown that *c9,t11* and CLA *t10,c12* have different effects on retinoid metabolism by feeding WT male mice for 4 weeks with enriched preparation of this two isomers. This study was able to show an uncoupling between the specific effects of these two distinct isomers on vitamin A metabolism (Ortiz et al. 2009). Specifically, only dietary CLA *t10,c12*, but not CLA *c9,t11*, increases serum levels of the retinol in wild-type mice. In addition, it has been shown that the levels of serum RBP were elevated only in CLA *t10,c12* fed animals (Figure 8).

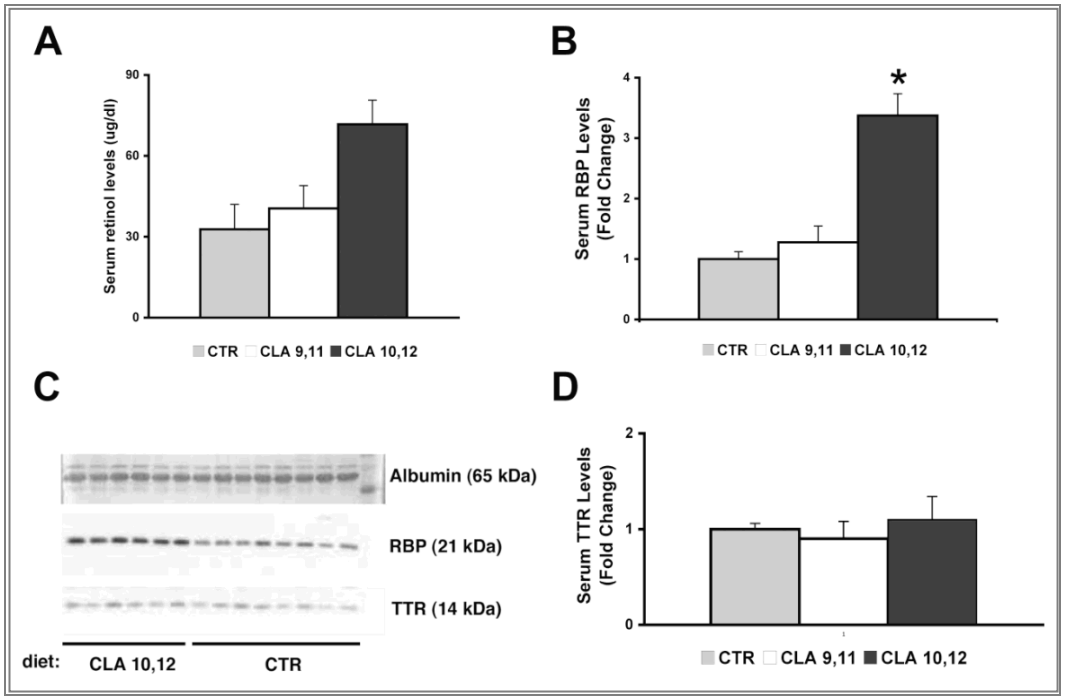

Figure 8. Serum levels of retinol, RBP, and TTR upon CLA consumption.

*Retinol levels were measured by reverse phase HPLC and RBP and TTR levels were measured by Western blot analysis. Five to six wild-type mice per group were analyzed at 14 weeks of age. (**A**) Serum retinol levels; (**B**) Serum RBP levels; (**C**) Representative western blot of serum RBP and TTR levels in mice fed CLA t10,c12; (**D**) Serum TTR levels. A rabbit polyclonal anti-rat RBP serum (60) and a rabbit polyclonal anti-rat TTR serum (61) were used for the western blot analysis. * indicates statistically significant difference between treatment and control diet group (p < 0.05).(Ortiz et al. JLR, 2009)*

The major site of synthesis and secretion of RBP is the hepatocyte, from where this protein mobilizes the retinoid stores (Soprano et al. 1994; Quadro et al. 2003). Therefore, they hypothesized that the excess ROH in the circulation of wild-type mice fed CLA *t10,c12* is secreted from the liver bound to RBP, i.e. that this isomer stimulates hepatic secretion of the complex ROH-RBP. The lack of changes in RBP mRNA levels and the increased levels of RBP protein in the liver of mice fed CLA *t10,c12* {Ortiz, 2009 #824} suggest that CLA may influence RBP secretion at a post-transcriptional level. The increase in LRAT mRNA levels in the liver of mice fed CLA *t10,c12* also suggests that RE accumulation occurs with this isomer

(Fig. 6). However, no change in hepatic RE concentration was observed upon CLA *t10,c12* feeding (Ortiz et al. 2009). This discrepancy in data is only apparent, since ROH and RE measurements reflect a steady state vitamin A status of the tissue and do not exclude a rapid turn over of RE accumulated in the liver, through secretion in the bloodstream of ROH bound to RBP. Retinoid oxidation is also increased in the liver of these mice (Ortiz et al. 2009).

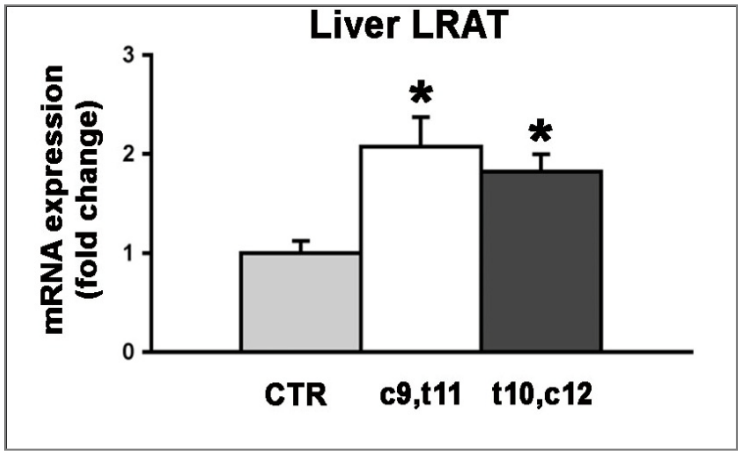

Figure 9. LRAT hepatic mRNA levels upon dietary CLA intake. *Measurements were performed by real-time RT-PCR analysis. Values are expressed as mean ± SE using the* $2^{-\Delta\Delta CT}$*. * indicates a statistically significant difference between treatment and control diet group (p < 0.05). (Ortiz et al. JLR, 2009).*

In the case of mice fed CLA c9,t11, the upregulation of the mRNA levels of LRAT in the liver (Fig. 9) and the increased concentration of hepatic RE (Ortiz et al. 2009) clearly demonstrated that the intake of this isomer induces hepatic RE accumulation. However, the lack of change in serum ROH and RBP levels (Figure 1) and hepatic RBP protein (Ortiz et al. 2009) in mice fed CLA *cis 9, trans11* indicate that retinoid accumulation in the liver stores is not followed by secretion of the ROH-RBP complex under this dietary regimen. Unlike the experiments performed with rats (9, 10), hepatic ROH levels did not increase upon CLA feeding in this study. The use of enriched preparations of isomers vs. preparations containing the same amounts of each isomer (9, 10, 62) could account for this discrepancy. Alternatively these could be species-specific differences in the response of the tissues to the CLA supplementation.

To rule out that the excess ROH in the circulation bound to RBP upon CLA *t10,c12* feeding was the result of vitamin A mobilization from the periphery of the body they analyzed the retinoid status of the adipose tissue, another important site of retinoid storage that expresses RBP (Janke et al. 2006). In wild-type mice, adipose RBP protein levels remained unaffected by CLA feeding with either isomer (Ortiz et al. 2009). In addition, only CLA *t10,c12* supplementation resulted in elevated levels of RE in fat tissue, with no changes in ROH concentration {Ortiz, 2009 #824}. These and other evidences (Ortiz et al. 2009) indicate that the CLA-stimulated secretion of ROH-RBP does not take place from fat and that it is the liver that redistributes the excess ROH bound to RBP towards the fat tissue, where it is stored as RE.

CLA feeding in humans alters serum retinol levels

Prof. S. Banni and colleagues investigated the effect of CLA feeding in humans. They analyzed serum retinol levels of 36 healthy volunteers (21 males and 15 males) who were fed with cheese enriched with CLA (CLA-enriched, 45 g/day) or Control cheese for 2 months in a double blind randomized cross over study. As shown in Table 1, the CLA-enriched cheese increased significantly circulating retinol levels (Data not published).

Table 1. Serum levels (nmoles/ml) of retinol and CLA. ** indicates p< 0.05*

DIET	Retinol	CLA
CONTROL	1.9 ± 0.9	15.3 ± 4.0
CLA-enriched	2.6 ± 1.1*	68.0 ± 29.0*

4. Study rationale and objectives

This study focuses on investigating previously un-noticed mechanisms of interactions between different types of fats, with special emphasis on conjugated linoleic acid (CLA), and vitamin A metabolism. Despite numerous studies performed in animal models and *in vitro* showing the health benefits of dietary CLA, its mechanisms of actions and metabolic implications still need to be further elucidated. Caution should be used in recommending CLA to prevent diseases in humans until these health effects are fully proven and the mechanisms of action entirely understood. Data from literature strongly indicates that dietary CLA can perturb vitamin A metabolism not only in rodent models, but potentially also in humans. Given this interference and given the fact that CLA controls many biological functions it is crucial to understand the mechanisms underlying this interaction, as we cannot rule out that changes in vitamin A metabolism could be responsible for both beneficial and/or detrimental effects of dietary CLA. Investigating the mechanistic effects of CLA on retinoid metabolism will help us advance our current understanding of some aspects of the vitamin A metabolism such as the mechanism regulating the secretion of hepatic ROH or those regulating dietary vitamin A absorption, that are still not fully clarified.

Specific Aim 1: to investigate the effects of dietary CLA on intestinal absorption and tissue uptake of retinoids.

The origin of the excess retinoids that accumulate in the liver of mice fed CLA remains unclear. Ingested vitamin A, regardless of its form, must first be emulsified to form micelles, then cross the brush-border membranes of the intestine, subsequently esterified and finally incorporated in chylomicrons and secreted into the lymph {Li, 2003 #820}. Literature data suggest that vitamin A absorption depends mainly on assembly and secretion of chylomicrons {Iqbal, 2009 #821; Harrison, 2005 #689}. Ingestion of dietary fat is the major determinant in the absorption of the vitamin A {Iqbal, 2009 #821; Harrison, 2005 #689}. Since CLA is a naturally occurring polyunsaturated fatty acid**, we have examined the hypothesis that dietary CLA may increase the intestinal absorption of retinoids by altering assembly and secretion of RE-containing chylomicrons.**

Specific Aim 2: to characterize the changes of vitamin A metabolism in response to an acute *versus* chronic administration of CLA and further investigate the effects of CLA intake on dietary vitamin A tissue distribution.

Approximately 66-75% of dietary retinoid is taken up and stored as retinyl ester in the liver, mainly in the nonparechymal hepatic stellate cells (also called Ito cells, lipocytes, and fat-storing cells). Only a smaller percentage of dietary retinoid is directly taken up by extrahepatic tissue (79). In times of insufficient dietary retinoid intake, retinol is secreted from the liver bound to RBP, its sole specific serum carrier, to be distributed to the target tissues (11). Data from the literature also suggest that newly absorbed retinoids can be re-secreted into the bloodstream from the liver to be transferred *via* RBP to the extrahepatic tissues (REF). A previous study (Ortiz et al. 2009) has shown that chronic CLA *t10,c12* feeding increases serum levels of the complex ROH-RBP without affecting RBP mRNA levels. Based on these evidences these authors have postulated that CLA *t10,c12* feeding stimulates the secretion of the complex ROH-RBP from the liver. **In this study we have investigated: A) The effects of a single acute administration of dietary CLA on RBP mediated hepatic retinol secretion and its redistribution towards peripheral tissues; B) How vitamin A metabolism changes in response to a chronic dietary supplementation of CLA, followed by an oral bolus dose of either CLA isomer.**

Specific Aim 3: to examine the effects of different types of oils on intestinal absorption of vitamin A.

Since the ingestion of dietary fats has the greatest impact on the absorption of the vitamin A we further investigate the effects of different dietary fats on vitamin A intestinal absorption. We tested olive oil, sunflower oil and flaxseed oil, that differ in fatty acid type content and we verify whether an acute administration of either of these three oils might perturb assembly and secretion of RE-containing chylomicrons.

5. Experimental Design

5.1 Acute dietary CLA administration

To start gaining insights on the effect of CLA intake on intestinal absorption of vitamin A we have performed this study with wild-type mice (C57Bl/6 x 129/sv). 14 week-old Wild-type and RBP-/- male mice maintained on a regular chow diet (containing 25 IU vitamin A/g diet) were intraperitoneally injected with P-407 (1 mg/g body weight) the evening before the experiment began, and then fed overnight. P-407 is a total lipase inhibitor that prevents hydrolysis and clearance of chylomicrons for 4-5 days, thus optimizing the chance of detecting vitamin A of intestinal origin in the serum of these mice. The following morning, mice were given a gavage dose containing one of the followings: A. $2x10^6$ cpm all-*trans*[^{3}H]ROH and 6ug of unlabeled all-*trans*-ROH in olive oil vehicle; B. $2x10^6$ cpm all-*trans*[^{3}H]ROH and 6ug of unlabeled all-*trans*-ROH in olive oil vehicle + 30 mg of CLA *c9,t11*; or C. $2x10^6$ cpm all-*trans*[^{3}H]ROH and 6ug of unlabeled all-*trans*-ROH in olive oil vehicle + 30 mg of CLA *t10,c12*. This amount of ROH corresponds to the amount of vitamin A consumed daily by our mice on a standard chow diet, as previously reported. The CLA dose corresponds to the amount of CLA consumed daily by mice receiving a diet supplemented with 1% CLA, as previously published {Ortiz, 2009 #824} [1% (10mg/g diet) x 3 (g of diet eaten in average by mice)= 30mg]. Finally, olive oil was chosen has a vehicle due to the similar metabolic fate of CLA and oleic acid.

Three hours after administration of the bolus dose of [^{3}H]ROH, when the concentration of newly absorbed dietary retinoids secreted in the bloodstream is at its peak, mice were sacrificed. Serum and tissues, including liver, adipose, heart, intestine were collected to measure their [3H]retinoid content by a scintillation counter (46) and their retinol and retinyl ester concentration by HPLC analysis.

To characterize the hepatic ROH, RE and [^{3}H] retinoid distribution, additional groups of mice, both wild-type and RBP -/-, were gavaged with the same mixtures of ROH and CLA described above (A, B or C) without P-407 administration prior to the gavage. This strategy will allow chylomicron clearance by the tissues.

5.2 Chronic CLA feeding and Chronic CLA feeding + gavage

Wild-type male mice (4-5 per group) were maintained on a regular chow diet from weaning. Starting at 10 weeks of age, the mice were randomly assigned to one of the following dietary regimens: A. purified (control) diet; B. control diet with 1% CLA c9,t11; C. control diet with 1% CLA t10,c12. Each diet provided 24.0%, 14.0% and 62.0% calories from protein, fat and carbohydrates, respectively, and contained 25 IU/g of vitamin A. Olive oil is the source of MUFA and the percentage of PUFA was maintained constant in the three diets. Note that the use of CLA preparations enriched in one of the two isomers (up to 75%) is considered a state-of-the-art methodology, given the prohibitive cost of performing chronic feeding experiments with individual isomers. Each group of mice were maintained on one of these diets for 4 weeks, i.e. until 14 weeks of age, when they were sacrificed. We chose the above-mentioned diets and length of the dietary treatment based on previously published results (Ortiz, 2009

Both diet and water were available to the animals on an *ad libitum* basis. Mice were maintained on a 12:12 light/dark cycle, with the period of darkness between 7:00 PM and 7:00 AM. All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Rutgers University Institutional Committee on Animal Care.

Additional groups of mice were maintained on the same dietary regimen described above and then subjected to three different gavage mixtures identical to those described above (A, B or C) They were sacrificed three hours after the administration of the bolus dose.

All mice used for these studies were sacrificed by CO2 inhalation. At the time of sacrifice, serum and tissues, including liver, intestine, adipose, heart, muscle, brain, were collected from the mice to measure retinol and retinyl ester concentration by HPLC analysis and [^{3}H] retinoid content by a scintillation counter.

5.3 Acute administration of different types of oils

14 week-old Wild-type and RBP-/- male mice maintained on a regular chow diet (containing 25 IU vitamin A/g diet) were intraperitoneally injected with P-407 (1 mg/g body weight) the evening before the experiment began, and then fed overnightThe following morning, mice were given a gavage dose containing one of the followings: A. $2x10^6$ cpm all-*trans*[^{3}H]ROH and 6ug of unlabeled all-*trans*-ROH in olive oil vehicle; B. $2x10^6$ cpm all-*trans*[^{3}H]ROH and 6ug of unlabeled all-*trans*-ROH in sunflower oil vehicle; or C. $2x10^6$ cpm all-*trans*[^{3}H]ROH and 6ug of unlabeled all-*trans*-ROH in flaxseed oil vehicle. This amount of ROH corresponds to the amount of vitamin A consumed daily by our mice on a standard chow diet, as previously reported. Three hours after administration of the bolus dose of [^{3}H]ROH, when the concentration of newly absorbed dietary retinoids secreted in the bloodstream is at its peak, mice were sacrificed. Serum and tissues, including liver, adipose, heart, intestine were collected to measure their [3H]retinoid content by a scintillation counter (46) and their retinol and retinyl ester concentration by HPLC analysis.

6. Materials and Methods

CLA Isomers

Isomers of CLA c9,t11 and t10,c12 (+98% pure) for gavage administration were purchased from Matreya (Pleasant Gap, PA). Enriched preparations of CLA isomers c9,t11 and t10,c12 for the diet, were obtained from Lipid Nutrition (Wormerveer, Netherlands). The diets were manufactured by Research Diets, Inc. as follows:

A. Purified control diet contained 29.5 gm% Soybean Oil, 16 gm% Olive Oil and 18.5 gm% Lard.

B. Purified control diet with 1% CLA c9,t11.

C. Purified control diet with 1% CLA t10,c12.

Each diet provided 24.0%, 14.0% and 62.0% calories from protein, fat and carbohydrates, respectively, and contained 25 IU/g of vitamin A.

Oils for gavage

Commercially available olive oil, sunflower oil and flaxseed oil were used for the gavage experiments.

HPLC analysis of retinoids

Reverse phase HPLC analysis was performed to measure serum and tissue retinol and retinyl esters levels (21). Retinoids were separated on a 4.6x250 mm Ultrasphere C18 column (Beckman, Fullerton, CA) preceded by a C18 guard column (Supelco Inc., Bellefonte, PA), using acetonitrile, methanol and methylene chloride (70:15:15, v/v) as the mobile phase flowing at 1.8 ml/min. A Dionex Ultimate 3000 HPLC system and a computerized data analysis workstation with the Chromeleon software were used. Retinol and retinyl esters were identified by comparing retention times and spectral data of experimental compounds with those of authentic standards. The concentrations of retinoids were determined by comparing peak integrated areas for unknowns against those of known amounts of purified standards. Loss during extraction was accounted for by adjusting for the recovery of retinyl acetate, the internal standard added immediately following homogenization of the tissues.

Analysis of triglyceride levels

Triglyceride levels were assayed by an enzymatic procedure using a commercial kit according to the accompanying instructions (Infinity Tryglicerides, Thermo Electron, Melbourne, Australia).

Western blot analysis

Serum content of RBP and TTR were analyzed by Western blot (27). A rabbit polyclonal anti-rat RBP antiserum (28), a rabbit polyclonal anti-rat TTR serum (29) were used for immunodetection. Signals were detected by using a Biorad Chemidoc XRS Molecular Imager System. Albumin, detected upon treating the membranes with either Coomassie or Ponceau S stain, was used as a loading control for serum samples. Albumin, detected by a rabbit polyclonal anti-Albumin antibody (Abcam), or Actin, detected by a mouse monoclonal anti-Actin antibody (Sigma), were used as a loading control for tissue samples. Themolecular weight of each detected protein is as follow: RBP, 21 kDa; TTR, 14 kDa; and Albumin, 65 kDa. The quantification of the membranes was completed by densitometry analysis with Quantity One Program (Biorad).

Total RNA extraction and real-time RT PCR analysis

Total RNA was extracted from tissue using RNA Bee (Tel-test Inc, TX) according to manufactures instructions. RNA concentrations were measured by spectrophotometry, followed by DNase I treatment (Roche Diagnostics, IN).

Two micrograms of the DNase Treated RNA was reverse transcribed to cDNA using instructions and reagents from Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics,IN). A no-reverse transcriptase control was included. For RT real-time PCR analysis, the Roche Applied Science Lightcycler 480 machine was used, together with the Lightcycler 480 SYBR green I master mix (Roche Diagnostics, IN). A series of experiments were performed to determine relative efficiency and concentrations for each primer of interest. Validation experiments were also performed to confirm that the housekeeping gene, □-Actin, was unaffected by the dietary treatments.

Primer sequences were as follows:

RBP – Fw 5'GGAGAACTTCGACAAGGCTC3'
Rev 5'CTGCACACACTTCCCAGTTG3'

LRAT– Fw 5'ATGAAGAACCCAATGCTGGAA3'
Rev 5'CTAATCCCAAGACAGCCGAAG3'

Actin – Fw 5'CGGAGGGAAAGATTCCTCTGGC3'
Rev 5'AGGGCCGGCACATTGAAGGTCT3'.

GAPDH – Fw 5'GACCCCTTCATTGACCTCAACTACATG3'
Rev 5'GTCCACCACCCTGTTGCTGTAGCC3'

MTTP – Fw 5'AGCTTTGTCACCGCTGTGC3'
Rev 5'TCCTGCTATGGTTTGTTGGAAGT3'

LDLr – Fw 5'GGATGTCGACTGTGTGTTGACG3'
Rev 5'GCACACTGGAATTCATCAGG3'

LRP – Fw 5'CCCTAAGGACCCCTGAAGAC3'
Rev 5'GGCCCGATACAACCAGTCTA3'

Final primer reaction conditions were as follows:

SYBR Green Master Mix, 500 nM Forward Primer , 500 nM Reverse Primer and PCR grade water. cDNA, no-reverse transcriptase control and a no template control were amplified using the following conditions: 95C for 10 min, followed by 40 cycles at 95C for 10 sec, 58C for 20 sec, 72C for 30 sec. This was followed by a dissociation curve to ensure the absence of any primer dimers. Furthermore, RT real-time PCR products were run on a gel to ensure the gene of interest was amplified. Each sample was run in triplicate and each RT real-time PCR run was repeated twice. Samples were analyzed by Relative Quantification, where the genes of interest are expressed relative to a chosen calibrator (control) using the ΔΔ CT method. This includes the subtraction of the CT value for the gene of interest from the CT value for the house keeping gene (β-Actin or GAPDH) to obtain ΔCT values. ΔΔCT values were obtained from subtracting ΔCT for each sample from the average ΔCT for the calibrator. The expression of each gene relative to the calibrator was calculated using 2(-ΔΔCT).

Statistical analysis

Normality of the data was determined using the Kolmogorov Smirnov test.
Non-normally distributed data were analyzed by a Kruskal-Wallis test followed by a Mann-Whitney test. Normally distributed data were analyzed by *t* test or ANOVA test with a correction for multiple comparisons using the Fisher's least significant test. Analyses were performed with SPSS statistical software (SPSS 11.0 for Windows Student Version, 2001; SPSS Inc IL). A p-value <0.05 was used to establish statistical significance.

7. Results – Specific aim 1

7.1 Effect of an acute dietary CLA administration on vitamin A intestinal absorption

In this part of the study triglyceride levels were measured in the serum of mice where the hydrolysis and the clearance of chylomicrons were pharmacologically inhibited. Then they were gavaged with an oral bolus of all-*trans*-[^{3}H] retinol (2× 10^{6} cpm) and unlabelled all-*trans*-retinol (6 µg) in 100 µl of olive oil or olive oil + either CLA isomer (70:30, v:v).

A group of mice maintained on a regular chow diet, injected with P-407 and not given a gavage administration was used as control group ("no gavage").

Triglyceride were elevated in the serum of mice given olive oil gavage compared to the "no gavage" group. TG concentration of the mice receiving an oral bolus of olive oil + either CLA isomer was similar to that of the "no gavage" group (Fig. 8)

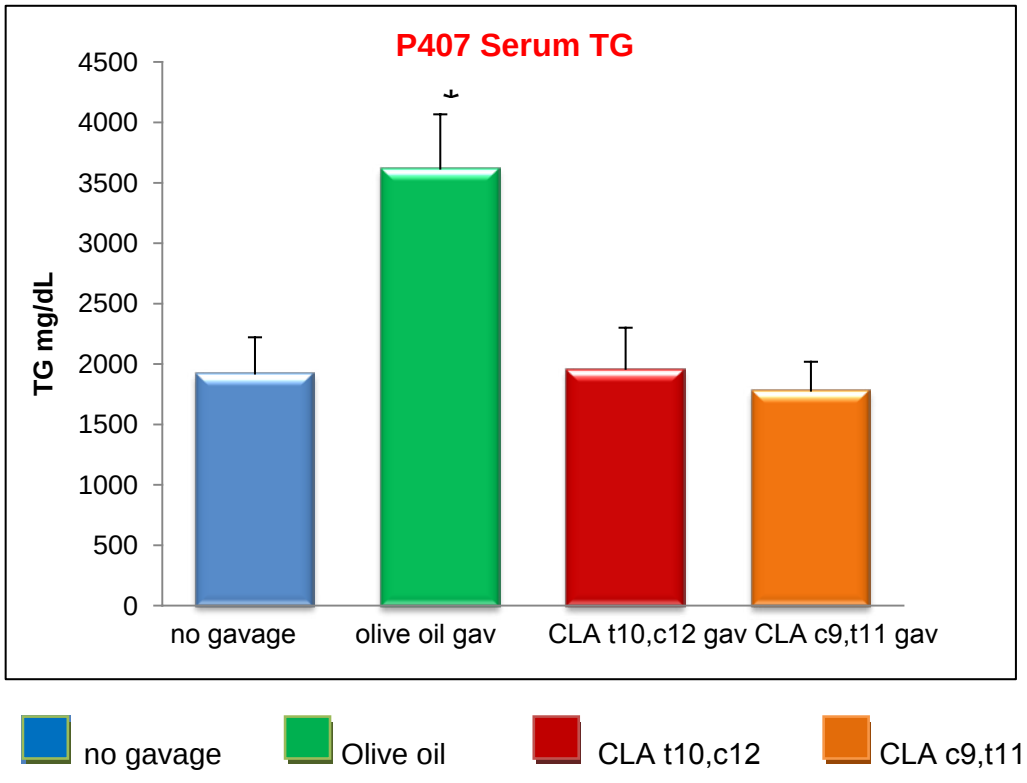

Figure 8: Triglyceride serum levels of mice injected with P-407

** indicates p < 0.05 versus no gavage. N= 4-5 each group*

To quantify the effect of an acute administration of CLA *t10,c12* and CLA *c9,t11* on serum retinol levels in mice, reverse phase HPLC analysis followed by fraction collections was performed. As shown in table 2, when the clearance of chilomycron remnants was inhibited by P-407, either CLA isomer induced a statistically significant elevation in circulating steady state levels of ROH and RE concentrations.

In figure 9 is shown an example of a serum HPLC profile from a mice injected with P-407, where different retinyl ester peaks are visible.

Table 2: Serum retinoids concentrations measured by HPLC. *Values are expressed as mean ± SE and normalized by TG levels * indicates p < 0.05 versus olive oil gavage. N= 4-5 each group*

GAVAGE	ROH/TG (µg/ul)	RE/TG (µg/ul)
OLIVE OIL	8.7 ± 0.4	290.9 ± 64.2
OLIVE OIL + CLA t10,c12 GAV.	20.5 ± 1.8 *	926.1 ± 142.1 *
OLIVE OIL + CLA c9,t11 GAV.	19.3 ± 1.9 *	583.9 ± 129.3 *

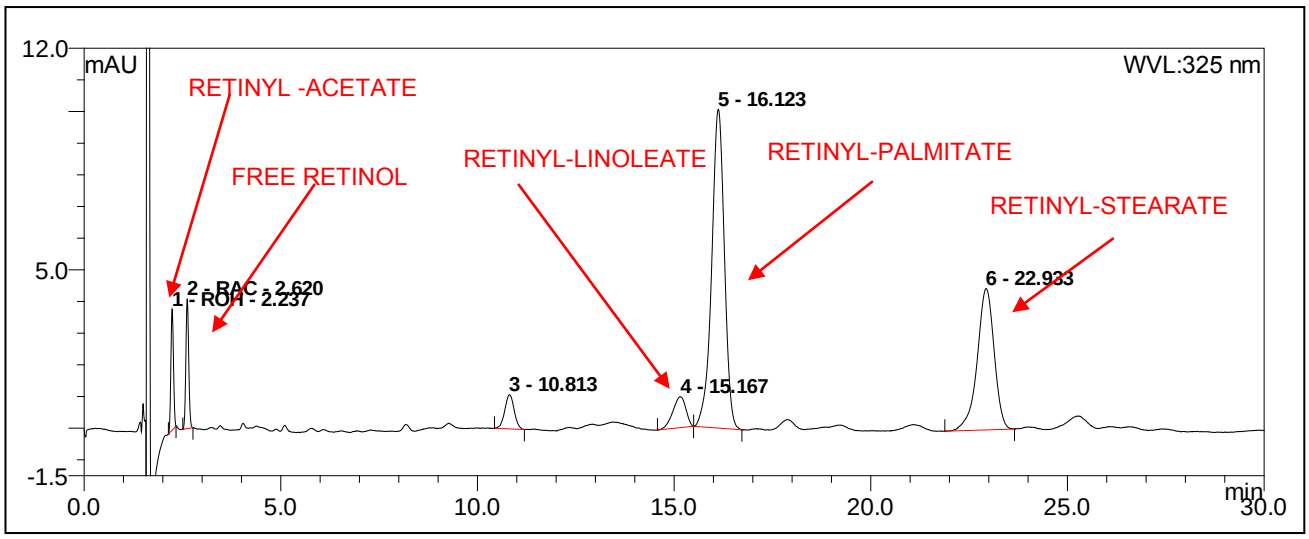

Figure 9. Serum HPLC profile of a mice injected with P-407. *Retinoids were separated on a 4.6x250 mm Ultrasphere C18 column (Beckman, Fullerton, CA) preceded by a C18 guard column (Supelco Inc., Bellefonte, PA), using acetonitrile, methanol and methylene chloride (70:15:15, v/v) as the mobile phase flowing at 1.8 ml/mi. Retinyl-acetate was used as internal standard.*

Cpm were measured by scintillation counting after collection of the different peaks with a fraction collector connected to the HPLC. We assessed levels of [^{3}H] retinoid present in the circulation, after a bolus dose containing tracer amounts of radiolabeled vitamin A. As shown in Figure 10A, upon P-407 administration, total [^{3}H] cpm (ROH +RE fractions) were elevated in the serum of mice gavaged with olive oil + either CLA isomer. Specifically, [^{3}H] cpm increased in the RE fraction, only when olive oil + CLA t10,c12 was given (Figure 10B).

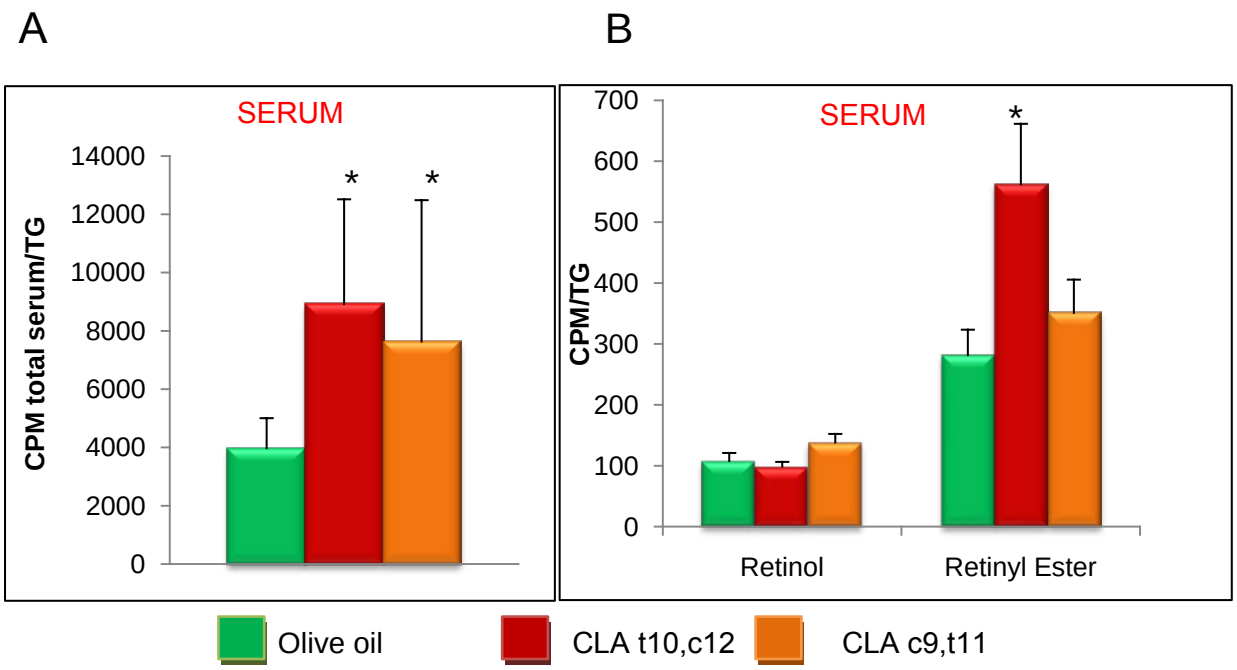

Figure 10. [^{3}H] counts/min in serum of mice injected with P-407 *CPM measured by scintillation counter in different retinoid fractions separated by reverse phase HPLC. Values are expressed as mean ± SE. Serum CPM are normalized by TG levels. * indicates p < 0.05 versus olive oil gavage. n=4-5 for each group.*

To verify whether the P-407 injection worked as an inhibitor of clearance of chylomicrons, total [^{3}H] counts/min were measured in the liver of mice gavaged with olive oil + either CLA isomer. As expected [^{3}H] total retinoid counts were reduced in P-407 injected groups, compared to non injected animals, thus proving that it inhibits hepatic clearance of recently ingested vitamin A (Figure 11).

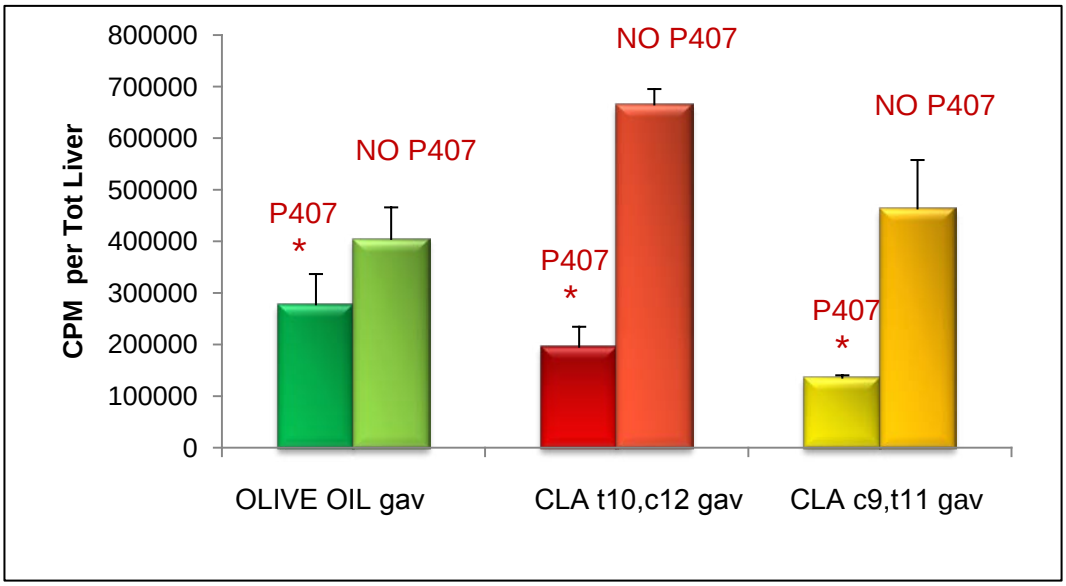

Figure 11. [^{3}H] counts/min in liver of mice injected with P-407 versus non injected with P-407 *CPM measured by scintillation counter in different retinoid fractions separated by reverse phase HPLC. Values are expressed as mean ± SE. * indicates p < 0.05 versus P-407 group. n=4-5 for* each group

Analysis of hepatic distribution of [^{3}H] retinoid have been performed. As shown in Figure 12, [^{3}H] ROH and to a lesser extent [^{3}H] RE were reduced when olive oil + either CLA isomer was given with P-407 injection, compared to olive oil gavage.

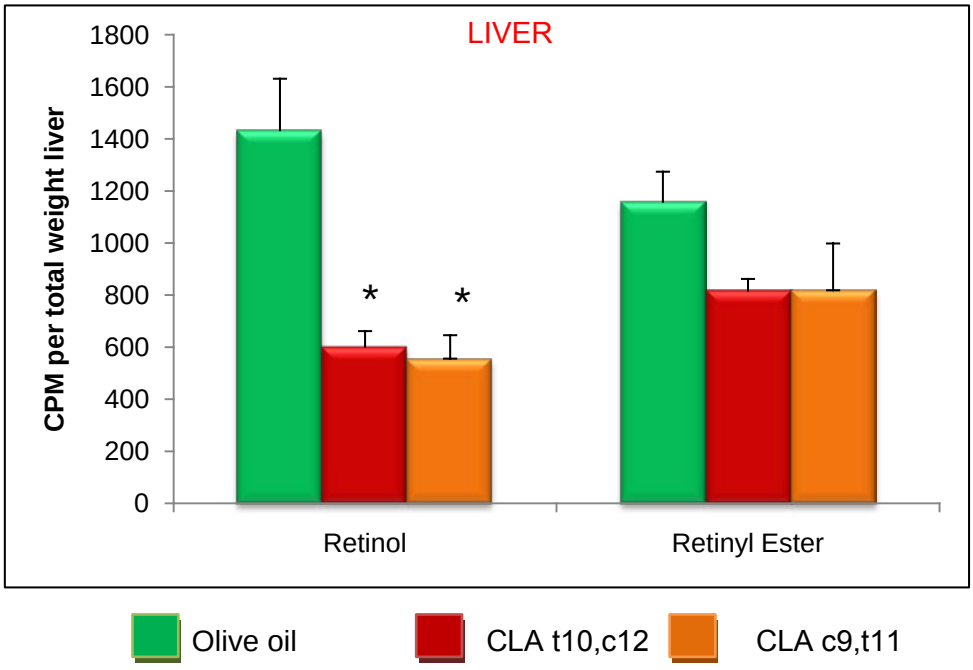

Figure 12. [^{3}H] ROH and [^{3}H] RE in liver of mice injected with P-407. *CPM measured by scintillation counter in different retinoid fractions separated by reverse phase HPLC. Values are expressed as mean ± SE. * indicates p < 0.05 versus olive oil gavage. n=4-5 for each group.*

We also analyzed cpm content of the intestine. Total [^{3}H]retinoid (ROH +RE fractions) were reduced in the small intestine of mice gavaged with olive oil + either CLA isomer (Figure 13). These results suggest that dietary CLA might have an effect in stimulating the intracellular processing of vitamin A in the enterocytes, thus reducing the [^{3}H] cpm in the small intestine. Overall, when the clearance of the chylomicrons is blocked, the increase in the radioactive counts in the serum and the decrease of the counts in the small intestine after a single administration of either CLA isomer, might suggests that CLA affects chylomicron formation and/or secretion.

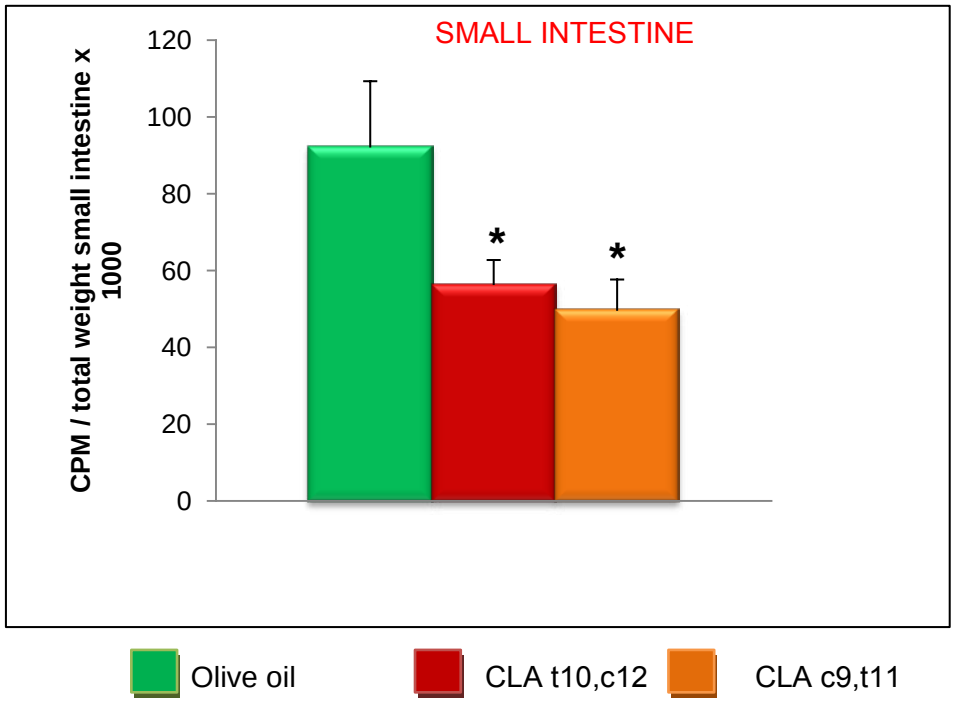

Figure 13. [^{3}H]-retinoid counts/min in small intestine of mice injected with P-407.

*CPM measured by scintillation counter in different retinoid fractions separated by reverse phase HPLC. Values are expressed as mean ± SE. * indicates p < 0.05 versus olive oil gavage. n=4-5 for each group. each group.*

Molecular mechanisms of intestinal chylomicron assembly

Real time RT-PCR analysis were performed on small intestine of the various groups of mice injected with P-407, to assess the expression levels of key players involved in the intestinal chylomicron assembly and/or secretion. To verify whether a single dose of dietary CLA would affect vitamin A incorporation in these particles, we measured mRNA expression levels of microsomal trygliceride transfer protein (MTTP) which transfers several lipids and assists in the formation of primordial apoB lipoprotein and LRAT , the key enzyme that esterifies ROH into RE.

As shown in Figure 14, the levels of MTTP were elevated, but not significantly, only in mice given CLA c9,t11 gavage and LRAT levels have the tendency to increase (not significantly, due to high standard deviation in olive oil group) when mice were given either CLA isomer.

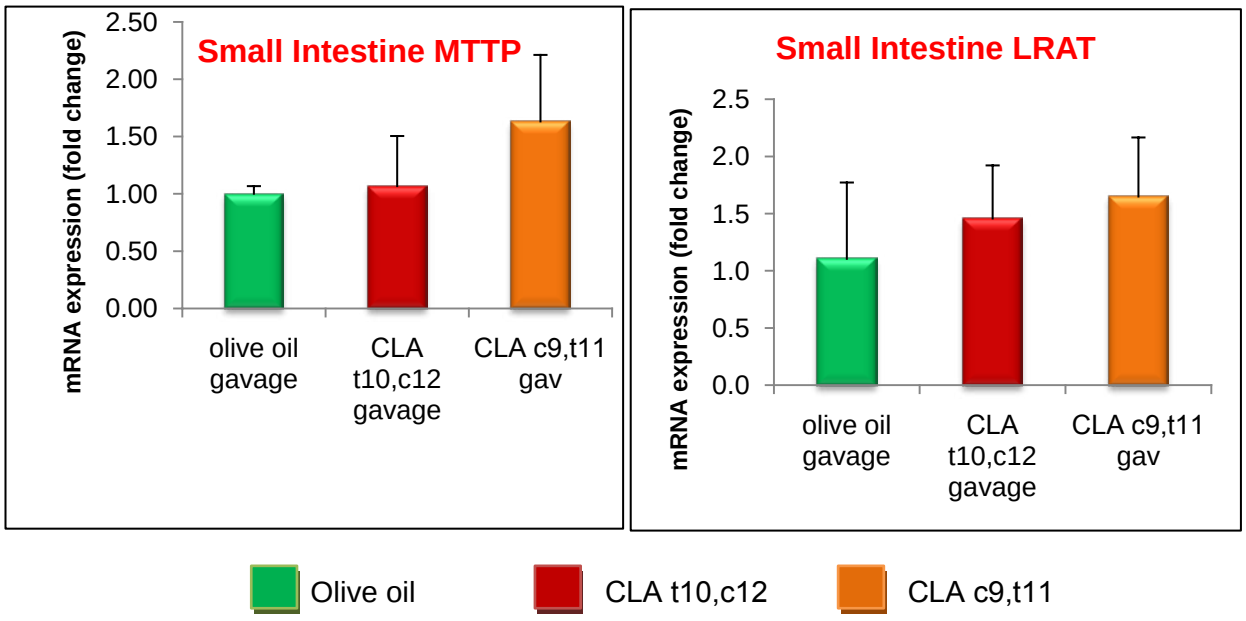

Figure 14. Small intestine mRNA expression levels of MTTP and LRAT measured by RT real-time PCR analysis. *Values are expressed as mean ± SD using the* $2^{-\Delta\Delta C}$

7.2 Tissue distribution of recently ingested vitamin A

Additional groups of mice were gavaged with the same mixtures of ROH and CLA described above (section 6: Materials and Methods) without P-407 administration prior to the gavage. To investigate the levels of [^{3}H]retinoid taken up by the liver, after an acute dose of CLA, Cpm analysis have been performed.

As shown in figure 15, [^{3}H]ROH and [^{3}H]RE counts were significantly increased in the liver of mice gavaged with either CLA isomer.

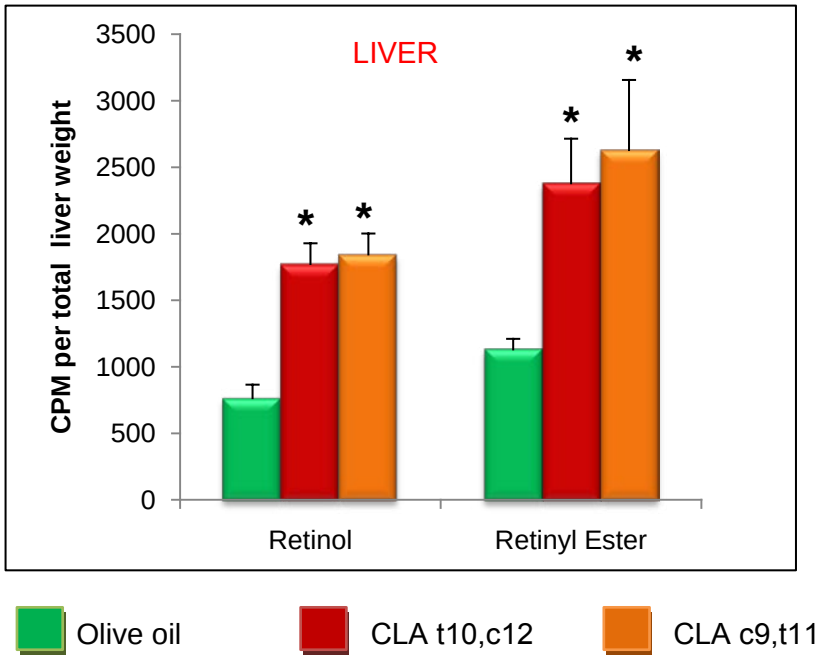

Figure 15. [^{3}H]retinoid levels in the liver of mice NON injected with P-407. *CPM measured by scintillation counter in different retinoid fractions separated by reverse phase HPLC Values are expressed as mean ± SE. * indicates p < 0.05 versus olive oil gavage. n=4-5 for each*

Then we investigated the effect of an acute administration of CLA *t10,c12* and CLA *c9,t11* on serum ROH levels in wild-type mice, by reverse phase HPLC analysis. Steady state serum ROH levels were not influenced by a single acute administration of CLA (Table 3)

Table 3. Steady state serum ROH concentrations upon a single CLA administration. Values are *expressed as mean ± SD. N= 4-5 each group*

GAVAGE	ROH /µg/dL
OLIVE OIL	35.9 ± 2.9
OLIVE OIL + CLA t10,c12 GAV.	35.9 ± 5.9
OLIVE OIL + CLA c9,t11 GAV.	46.1 ± 3.4

We also measured the levels of [^{3}H]retinoid in the serum, to follow the turnover of recently ingested radiolabeled vitamin A. Cpm analysis show higher levels of total serum [^{3}H] cpm in mice gavaged with Olive oil + either isomer. Specifically, [^{3}H] cpm increased in the serum ROH fraction. (Figure 16 A-B). To verify whether this increase in [^{3}H] cpm after a single administration of CLA was due to an increased secretion of the complex ROH-RBP from the liver, the same type of experiment has been performed, using RBP -/- mice. As shown in Figure 15 C, in the absence of RBP, [^{3}H] cpm, both in the ROH and RE fraction, don't change among groups, thus supporting the hypothesis that CLA acute feeding enhances hepatic ROH uptake and likely its resecretion into the bloodstream.

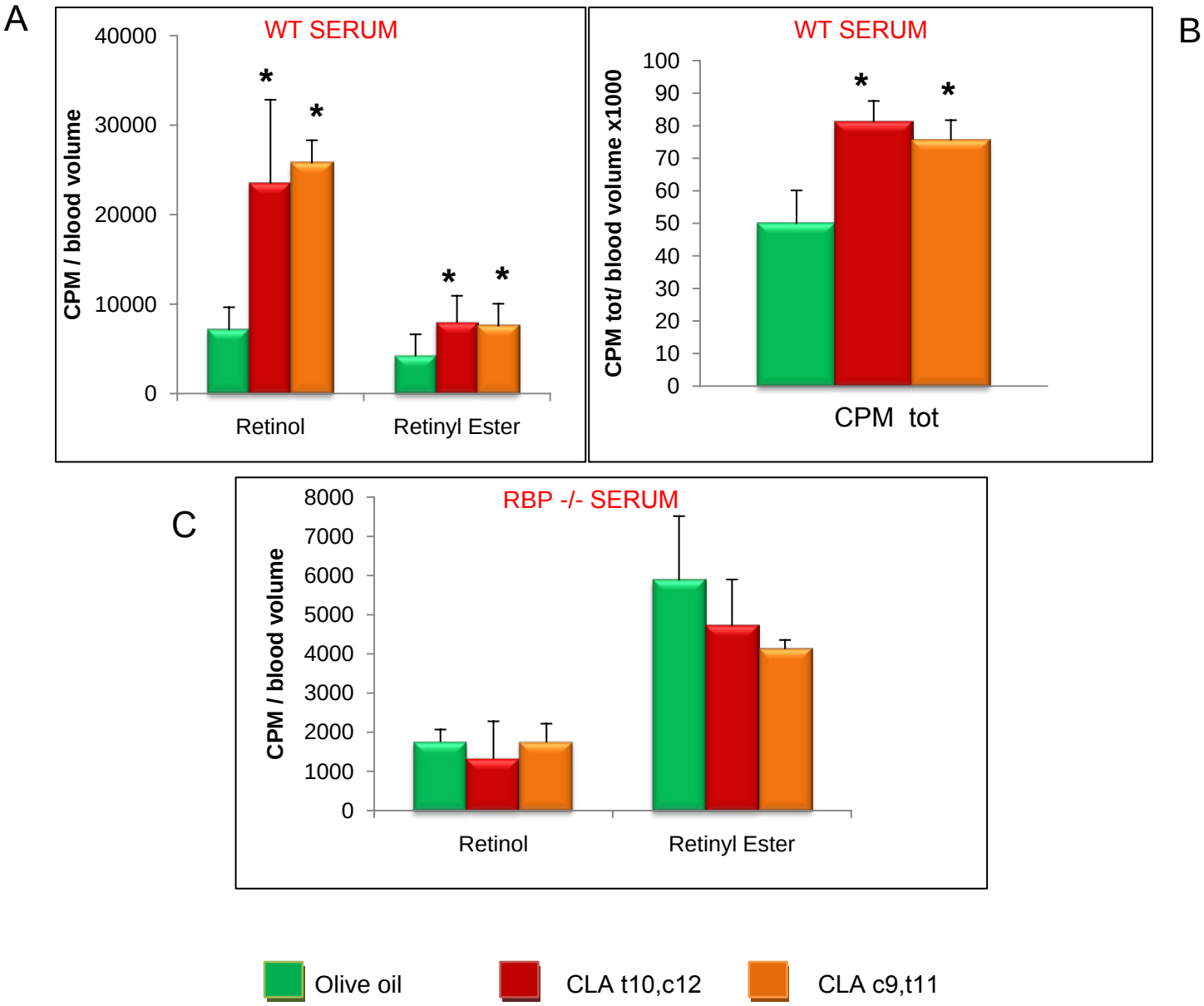

Figure 16. [^{3}H]retinoid levels in serum of mice NON injected with P-407. *CPM measured by scintillation counter in different retinoid fractions separated by reverse phase HPLC* **(A).** [^{3}H] ROH and [^{3}H] RE levels in wild-type mice**. (B).** *total* [^{3}H]retinoid levels (ROH + RE fraction) in wild-type mice. **(C).** [^{3}H] ROH and [^{3}H] RE levels in RBP -/- mice. *Values are expressed as mean ± SE.*
** indicates $p < 0.05$ versus olive oil gavage. n=4-5 for each group.*

To gain insights into whether an acute CLA administration is able to alter retinoid metabolism of other tissues in the body, [^{3}H] cpm were measured also in some important peripheral tissues, such as adipose tissue and small intestine. As shown in Figure 17, [^{3}H]retinoid were elevated in both these two districts, after gavage with either CLA isomer, demonstrating that there is an increased uptake of [^{3}H]ROH in peripheral tissues at least in WT mice able to mobilize the hepatic retinoid reserves *via* RBP*.*

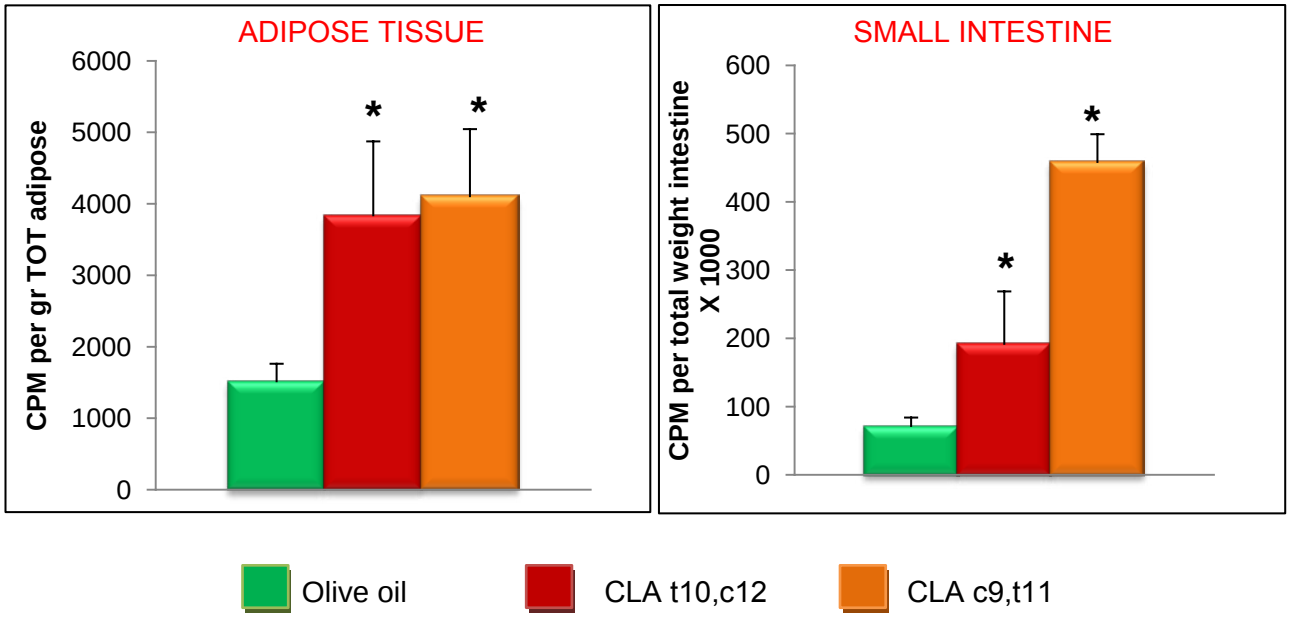

Figure 17. [^{3}H]retinoid levels in adipose and small intestine of mice NON injected with P-407. *Values are expressed as mean ± SE. * indicates p < 0.05 versus olive oil gavage . n=4-5 for each group*

Molecular mechanism of hepatic uptake of chylomicron remnants

To investigate the mechanism that leads to increased [^{3}H]retinoid in the liver, after an acute CLA administration, real time RT-PCR analysis were performed to assess the expression levels of some of the receptors involved in the uptake of chylomicron remnants, such as LDLr (Low density lipoprotein receptor) and LRP (LDL receptor related protein).

As shown in Figure 18, the mRNA expression levels of LRP were significantly downregulated in mice given an oral bolus of either CLA isomer. LDLr mRNA levels also showed a trend toward a reduction that reached statistical significance only upon administration of the CLA *c9,t11* gavage.

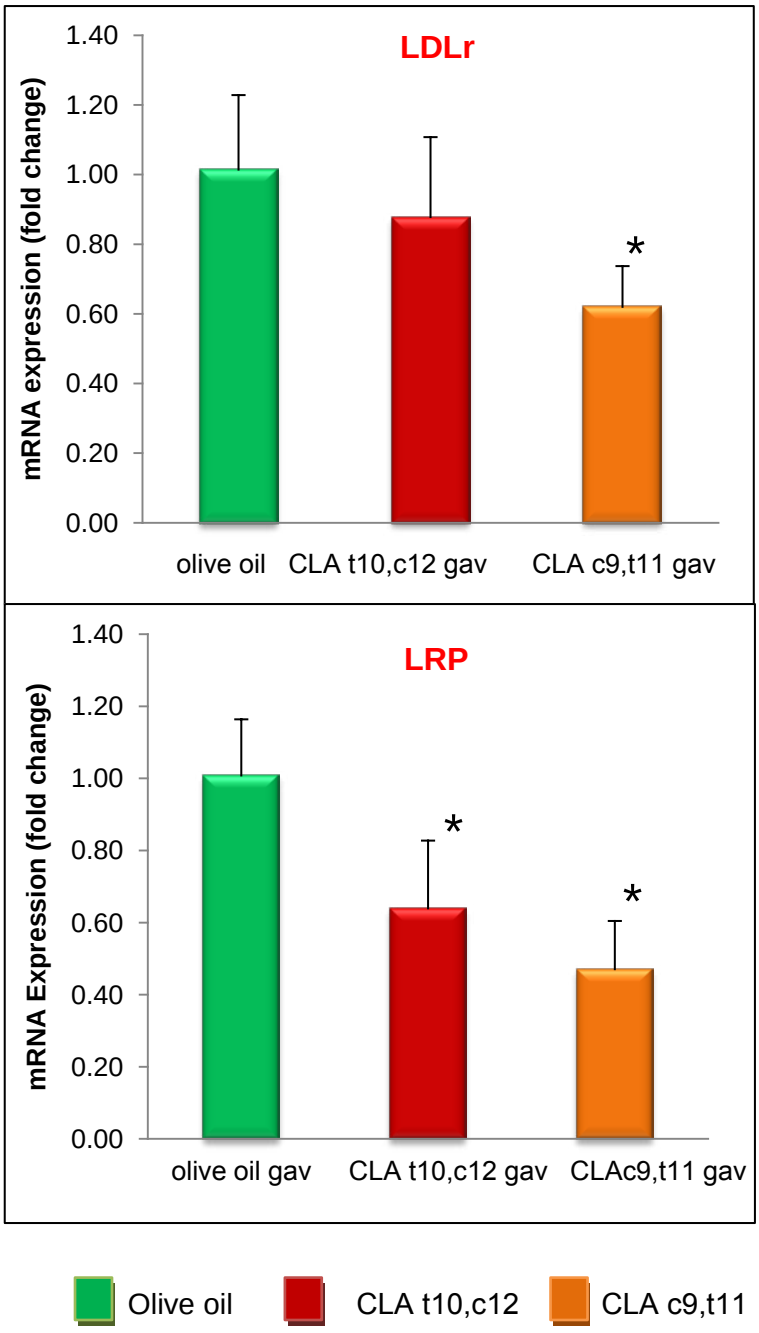

Figure 18. Liver mRNA expression levels of LRP and LDLr measured by RT real-time PCR analysis. *Values are expressed as mean ± SD using the $2^{-\Delta\Delta CT}$. * indicates $p < 0.05$ versus olive oil gavage. n=3-4 for each group*

The complex retinol-RBP is secreted from the liver to meet the vitamin A requirements of the periphery of the body (11, 12). Therefore, we set out to investigate whether CLA *t10,c12* and CLA *c9,t11* isomers affect mRNA expression levels of RBP in the liver, after a gavage administration. As shown in Figure 19, mRNA levels of hepatic RBP measured by RT real-time PCR analysis were not affected by the acute CLA dietary supplementation.

To further investigate the correlation between intake of CLA and increased hepatic [^{3}H]retinoids, we next measured the mRNA levels of the main enzyme that esterifies retinol into retinyl ester, the storage form of retinoids: lecithin:retinol acyltransferase (LRAT). Only upon CLA c9,t11 gavage, hepatic levels of LRAT mRNA showed a trend toward an increase when compared to an acute administration with olive oil.

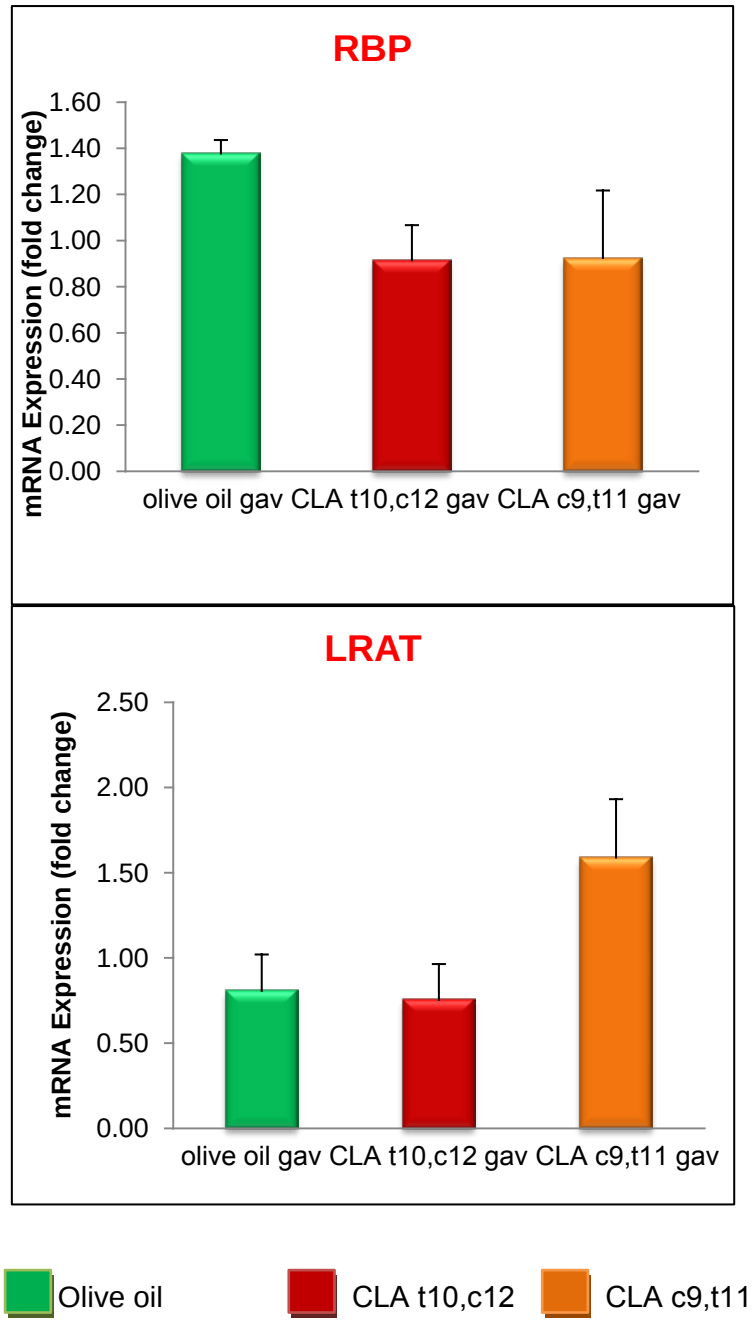

Figure 19. Liver mRNA expression levels of RBP and LRAT measured by RT real-time PCR analysis. *Values are expressed as mean ± SD using the* $2^{-\Delta\Delta CT}$*. n=3-4 for each group*

Results – Specific aim 2

7.3. Effect of a chronic dietary CLA intake on circulating retinol and RBP levels

In this part of the study we investigated the effects of a purified diet supplemented with 1% CLA *t10,c12* or 1%CLA *c9,t11* on circulating levels of ROH and RBP . As shown in figure 20, our results demonstrated that CLA *t10,c12* is the only isomer that is able to enhance serum levels of ROH and its specific carrier, RBP, thus confirming previously published data by Ortiz et al (Ortiz, Wassef et al.,2009), where the same effect was seen in mice fed with a regular chow diet (not purified) supplemented with 1% CLA *t10,c12* or 1%CLA *c9,t11.*

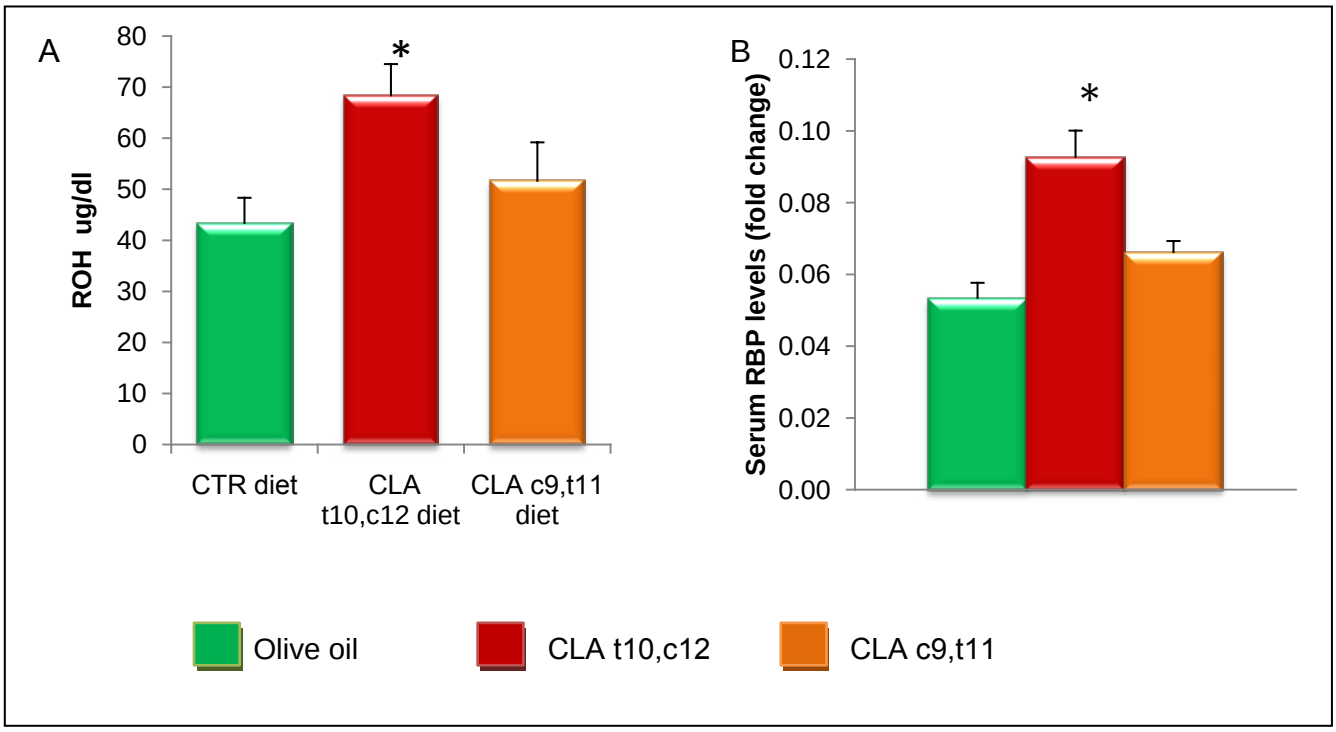

Figure 20. Serum levels of ROH and RBP upon CLA consumption. *ROH and RBP levels were measured by reverse phase HPLC and Western blot analysis, respectively. Four to five wild-type male mice per group were analyzed after 4 weeks of CLA feeding (See section n.6 for detailed description of the diets) (**A**) Serum retinol levels; (**B**) Densitometry analysis of serum RBP levels. A rabbit polyclonal anti-rat RBP (60) was used for the western blot analysis. * indicates a statistically significant difference between treatment and control diet group ($p < 0.05$).*

7.4. Effect of an acute dietary CLA intake on vitamin A tissue distribution

We next verified whether an acute administration of either dietary CLA isomers would affect the steady state retinoid levels in the serum of mice fed with one of the three dietary regimen described above (*See materials and methods: section n.6).*

The analysis of the steady state levels of retinol in the serum of mice chronically stimulated to purified supplemented diets and then subjected to gavage administrations showed that the acute intake of either CLA isomer seemed to blunt the differential effects of each isomer, observed in mice maintained upon chronic feeding (Table 4 A-B). In fact, ROH levels are the same among the groups.

In addition to this experiment, we performed Western Blot analysis to measure the levels of retinol binding protein (RBP) in the serum. Circulating RBP protein level were also blunted by the effect of the gavage administrations, showing the same protein levels among groups.

Table 4. Steady state serum levels of ROH upon CLA consumption. *Serum retinol levels (µg/dl) were determined by reverse phase HPLC. Wild-type (WT) male mice were maintained on three different dietary regimens for four weeks.* ***(A)*** *Serum ROH levels of mice fed for four weeks.* ***(B)*** *Serum ROH levels of mice subjected to three different gavage mixtures after four weeks of chronic supplementation. Values are expressed as mean ± SD. N= 4-8 each group. . * indicates a statistically significant difference between treatment and control diet group (p < 0.05).*

.

A

4 weeks diet	ROH /µg/dL
CTR	43.4 ± 4.9
CLA t10,c12	68.4 ± 6.1 *
CLA c9,t11	51.7 ± 7.5

B

4 weeks diet + gavage	ROH /µg/dL
CTR + olive oil gav.	53.9 ± 8.5
CLA t10,c12 +CLA t10,c12 gav.	59.4 ± 9.3
CLA c9,t11 + CLA c9,t11 gav.	59.6 ± 12.3

We also measured the levels of [^{3}H]retinoid in the serum, to follow the turnover of recently ingested radiolabeled vitamin A. Cpm analysis showed that total [^{3}H] retinoid (ROH +RE fractions) were significantly reduced in the serum of mice gavaged with olive oil + either CLA isomer. Specifically, [^{3}H] cpm levels decreased in the ROH fraction (Figure 21).

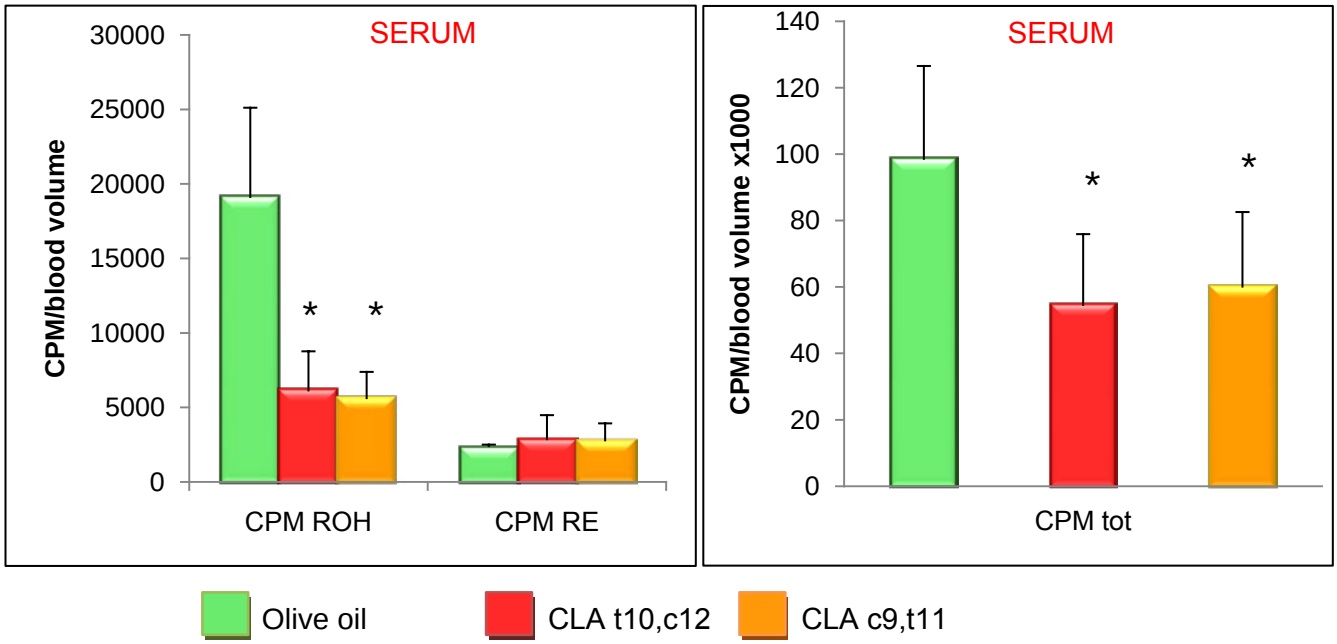

Figure 21. [^{3}H]retinoid levels in serum of mice subjected to three different gavage mixtures after four weeks of chronic supplementation. *CPM were measured by scintillator counter after fractionation with a fraction collector connected to HPLC. Values are expressed as mean ± SD. * indicates p < 0.05 versus olive oil gavage . n=4-7 for each group.*

I've next quantify the effect of chronic dietary CLA *t10,c12* and CLA *c9,t11* on liver retinol and retinylester levels by reverse phase HPLC. We noticed that RE levels were significantly higher in mice given different types of gavage mixture, compared to those not given the gavages. Specifically RE levels increased approximately 2-fold and more when olive oil gavage or CLA *t10,c12* were administered after a chronic feeding, compared to those only fed with a chronic diet. These data might suggest a fatty acid specific effect on vitamin A uptake from the liver.

As shown in Table 5 A and B, in both sets of experiments, only CLA *t10,c12* feeding significantly decreased RE concentrations. These results, along with those from the serum and RBP protein analysis, are consistent with published data that demonstrated that only CLA *t10,c12* stimulates secretion of the complex ROH-RBP from the liver, thus resulting in increased serum levels of ROH and its specific carrier (RBP). (Ortiz, Wassef et al. 2009).

Table 5. Steady state hepatic levels of ROH upon CLA consumption. *Liver ROH and RE levels (µg/g) were determined by reverse phase HPLC. Wild-type (WT) male mice were maintained on three different dietary regimens for four weeks.* ***(A)*** *Hepatic ROH and RE levels of mice fed for four weeks.* ***(B)*** *Hepatic ROH and RE levels of mice subjected to three different gavage mixtures after four weeks of chronic supplementation. Values are expressed as mean ± SD. N= 4-7 each group. . * indicates a statistically significant difference between treatment and control diet group (p < 0.05)*

A

4 weeks diet	ROH (ug/g)	RE (ug/g)
CTR	8.8 ± 3.6	63.4 ± 6.3
CLA t10,c12	7.7 ± 4.1	32.2 ± 6.5 *
CLA c9,t11	4.5 ± 1.1	83.6 ± 32.6

B

4 weeks diet + gavage	ROH (ug/g)	RE (ug/g)
CTR + olive oil gav.	5.3 ± 1.6	120.1 ± 19.1
CLA t10,c12 + CLA t10,c12 gav.	3.1 ± 1.1 *	83.1 ± 26.6 *
CLA c9,t11 diet + CLA c9,t11 gav.	7.1 ± 3.5	108.1 ± 34.6

We have then analyzed [^{3}H] retinoid levels in the liver to see the effect of a single dietary CLA intake on the rapid turnover of recently ingested vitamin A. [^{3}H]retinoid levels (ROH +RE fractions) were significantly reduced in the liver of mice supplemented and gavaged with CLA *t10,c12* isomer and to a lesser extend with CLA *c9,t11*.Specifically, [^{3}H] cpm decreased in the RE fraction. (Figure 22).

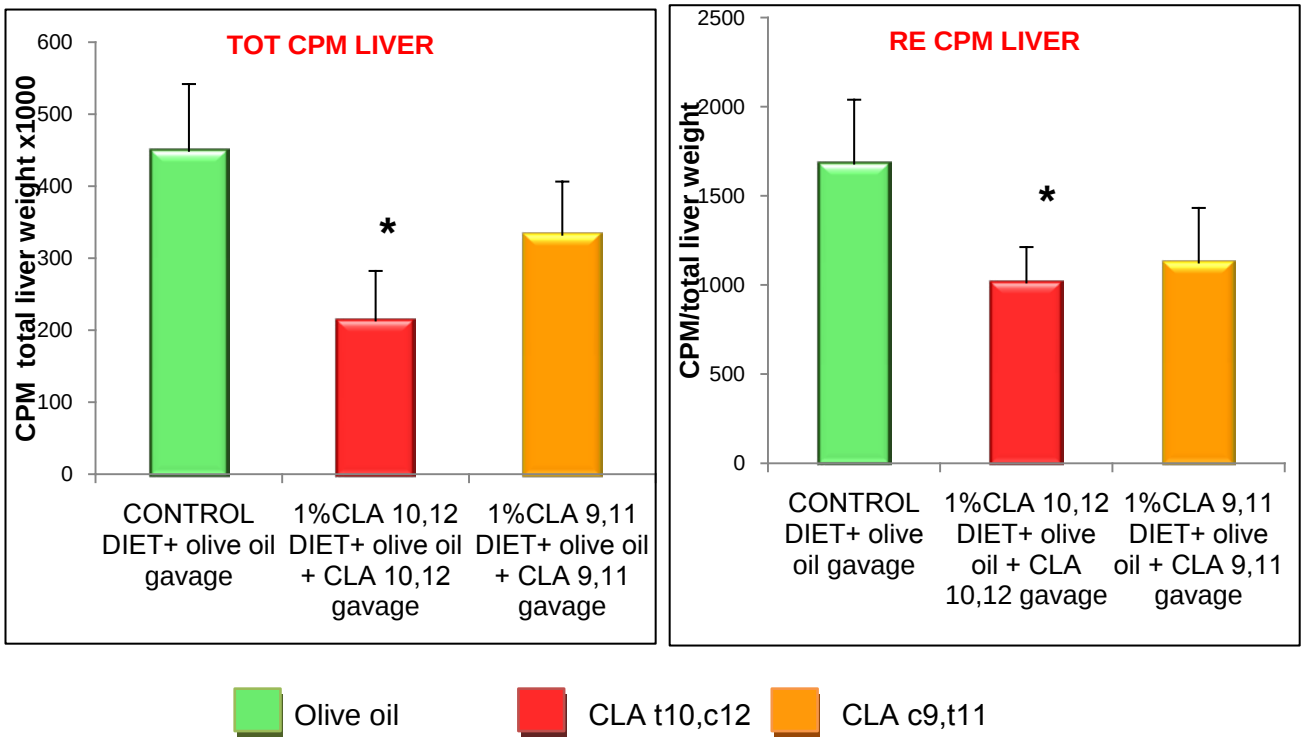

Figure 22. [^{3}H]retinoid in the liver of mice chronic supplemented and gavaged with CLA isomers. *Values are expressed as mean ± SD. * indicates p < 0.05 versus olive oil gavage . n=4-7 for each group*

Finally we measured [^{3}H] retinoid content in the adipose tissue: another important storage tissue for retinoid. Total [^{3}H] cpm (ROH +RE fractions) were significantly reduced only when mice were fed with 1%CLA t10,c12 diet + CLA t10,c12 gavage, compared to control group (Figure 23).

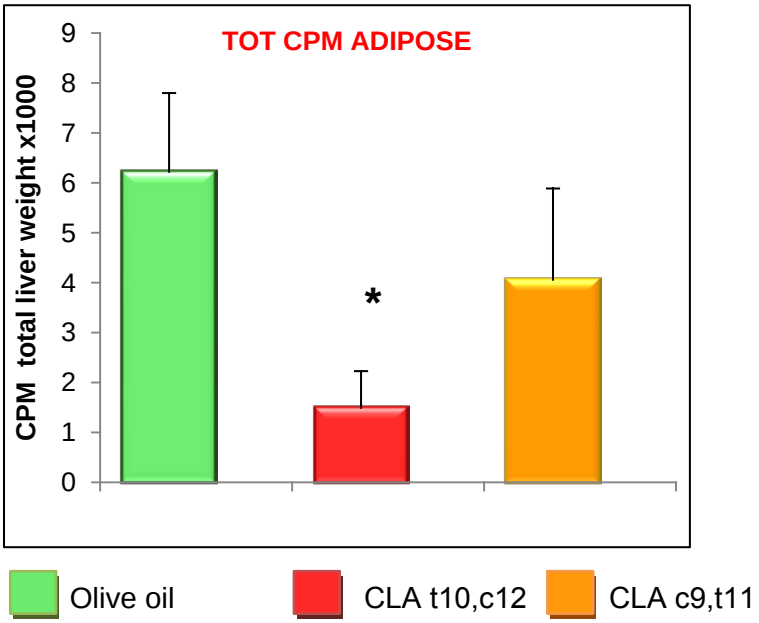

Figure 23. [^{3}H]retinoid in the adipose tissue of mice chronic supplemented and gavaged with CLA isomers. *Values are expressed as mean ± SD. * indicates p < 0.05 versus olive oil gavage . n=4 for each group*

Results – Specific aim 3

7.5 Effect of an acute dietary intake of Sunflower oil, Flaxseed oil and Olive oil on vitamin A intestinal absorption

In this part of the study, we investigated the effect of an acute administration of different types of oils on vitamin A intestinal absorption. We analyzed the triglyceride levels in the serum of mice where the hydrolysis and the clearance of the chylomicrons were pharmacologically inhibited by injection with P-407. Then, they received a gavage with a single oral bolus of all-*trans*-[^{3}H] retinol (2× 10^{6} cpm) and unlabelled all-*trans*- retinol (6 µg) in:

- 100 µl of olive oil
- 100 µl of sunflower oil
- 100 µl of flaxseed oil

A group of mice injected with P-407 and not given a gavage administration was used as control group (“no gavage”).

As shown in Figure 24, triglyceride levels were significantly elevated in the serum of mice gavaged with olive oil and with sunflower oil. Flaxseed oil gavage didn’t induce any significant change compared to “no gavage” group.

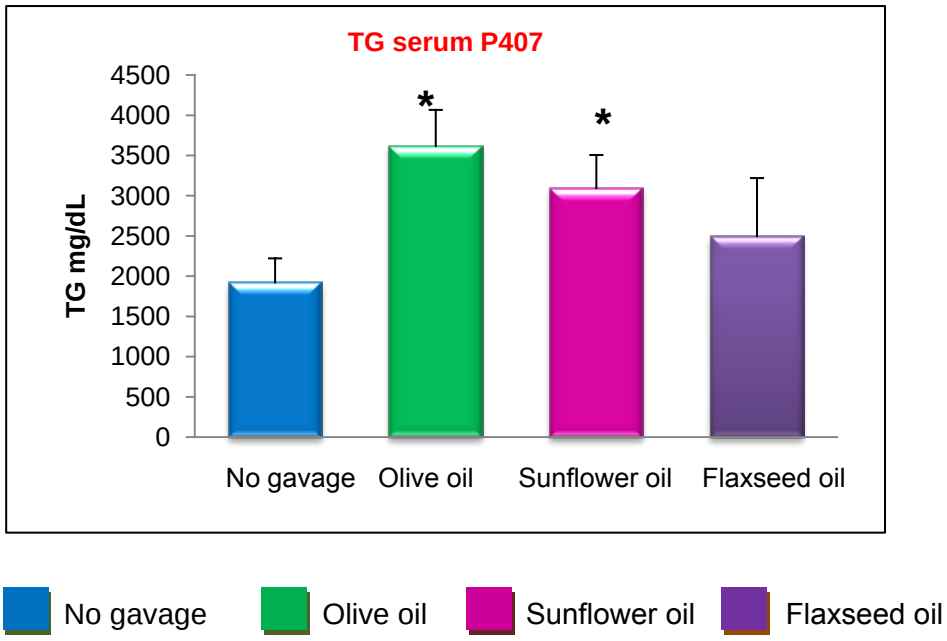

Figure 24. Triglyceride serum levels of mice given an acute dose of different oils. ** indicates p < 0.05 versus no gavage. N= 4-5 each group*

To quantify the effect of an acute administration of olive oil, sunflower oil and flaxseed oil on serum retinol levels in mice, reverse phase HPLC analysis followed by fraction collections have been performed. As shown in table 6, olive oil and sunflower oil induced a statistically significant reduction in circulating ROH concentrations. RE concentration is also decreased when olive oil and flaxseed oil are acutely administered, compared to “no gavage” group.

Table 6: Steady state serum retinoid concentrations measured by HPLC. Values are *expressed as mean ± SE and normalized by TG levels * indicates p < 0.05 versus olive oil gavage. N= 4-5 each group*

GAVAGE	ROH/TG (µg/ul)	RE/TG (µg/ul)
NO GAVAGE	16.9 ± 4.1	500.1 ± 147.2
OLIVE OIL	8.73 ± 0.8 *	292.6 ± 125.7 *
SUNFLOWER OIL	7.8 ± 1.5 *	370.5 ± 243.8
FLAXSEED OIL	15.5 ± 4.1	185.1 ± 68.4 *

We also measured [^{3}H]retinoid levels in the circulation. As shown in figure 22, [^{3}H] cpm were reduced, specifically in the ROH fraction in mice gavaged with sunflower oil and flaxseed oil, compared to olive oil and no significant change was noticed in the RE fraction, probably due to high standard deviation. In contrast, tot [^{3}H] cpm (ROH + RE fractions) were significantly increased with flaxseed oil gavage (Figure 25).

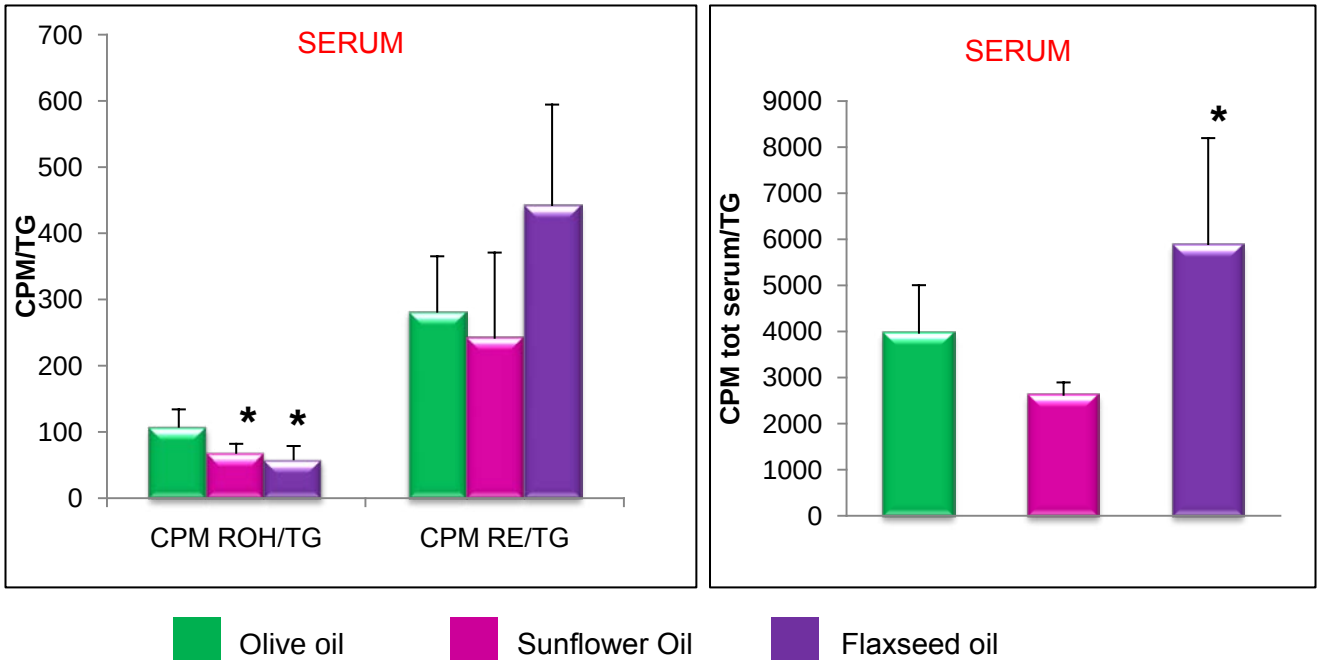

Figure 25. [^{3}H] retinoid levels in serum of mice given an acute dose of olive oil, sunflower oil or flaxseed oil. *CPM measured by scintillation counter in different retinoid fractions separated by reverse phase HPLC. Values are expressed as mean ± SD. Serum CPM are normalized by TG levels. * indicates p < 0.05 versus olive oil gavage. n=4-5 for each group.*

To further investigate the effects of different oils on the intestinal absorption of vitamin A, we analyzed the steady state retinoid levels in the small intestine of these mice.

HPLC analysis revealed that ROH levels are decreased in mice given sunflower oil and flaxseed oil gavage, compared to olive oil (Table 7).

Table 7: Steady state small intestine retinoid concentrations measured by HPLC. Values are *expressed as mean ± SD * indicates p < 0.05 versus olive oil gavage. N= 4-5 each group*

GAVAGE	ROH (ng/g)	RE (ng/g)
OLIVE OIL	1814.1 ± 444.1	866.9 ± 235.4
SUNFLOWER OIL	1132.4 ± 257.1 *	934.1 ± 206.1
FLAXSEED OIL	1226.2 ± 679.8	1296.5 ± 364.4

[^{3}H] retinoid distribution in the small intestine was also analyzed. Cpm measurements showed a reduction in the [^{3}H]ROH levels, when mice were given either sunflower oil or flaxseed oil. A significant decrease in [^{3}H] cpm was also shown in the RE fraction, but only with sunflower oil gavage, compared to olive oil (Figure 26).

Overall, these results suggest that an acute administration of olive oil, sunflower oil or flaxseed oil can perturb vitamin A metabolism. Specifically the increase in total [^{3}H] retinoid levels in the serum is induced more clearly by flaxseed oil, rather than sunflower oil and the decrease in the small intestine is induced by both of them, compared to olive oil.

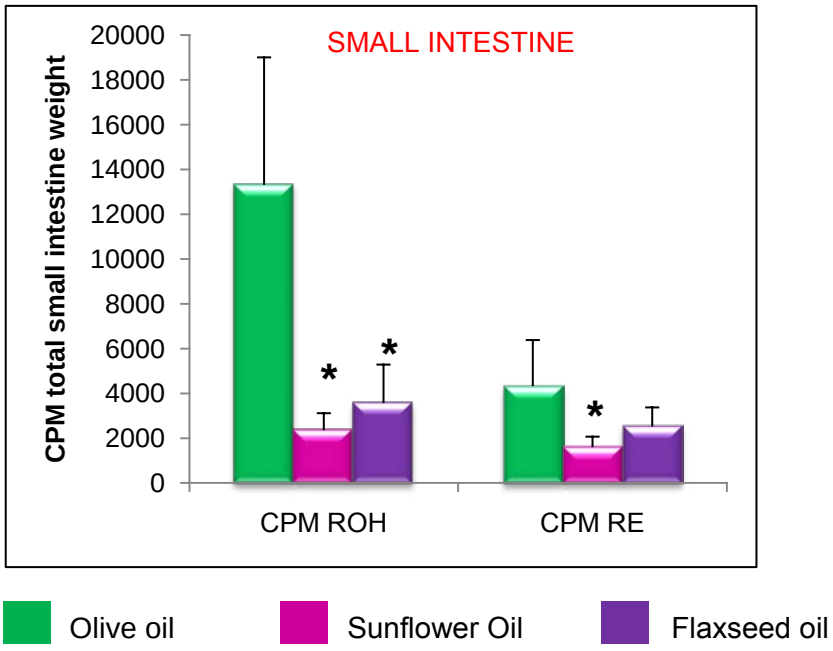

Figure 26. [^{3}H] retinoid levels in the small intestine of mice given an acute dose of olive oil, sunflower oil or flaxseed oil. *CPM measured by scintillation counter in different retinoid fractions separated by reverse phase HPLC. Values are expressed as mean ± SD. * indicates p < 0.05 versus olive oil gavage n=4-5 for each group.*

8. Discussion

In omnivores (including humans), the two dietary sources of vitamin A are preformed vitamin A (from animal sources) or provitamin A carotenoids (from plant sources), which are subsequently converted in the body to retinal and retinol. Dietary fat facilitates the utilization of vitamin A and, based on serum retinol responses following ingestion of meals containing carotene and fat sources, it has been reported that the amount of fat required in a meal may be minimal (3–5 g) (Ribaya-Mercado 2002).

The rate limiting steps in the lymphatic absorption of vitamin A involve intracellular processing of vitamin A within the enterocyte. The ingestion of dietary fat, a major determinant in the absorption of preformed vitamin A and provitamin A carotenoids, can potentially affect lymphatic absorption of vitamin A at several steps:

1. Solubilization of vitamin A in mixed micelles, which is needed for efficient uptake by the enterocyte.
2. Esterification of retinol with long chain fatty acids.
3. Incorporation of retinyl esters into chylomicrons
4. Secretion of chylomicrons into the lymph.

The key steps appear to be related to chylomicron formation and secretion and are closely coupled with fat absorption (Li et al. 2003).

The results of studies in humans examining the short-term effects of dietary fatty acids on postprandial lipemia suggest that the fatty acid composition of the test meal may influence the absorption, synthesis and secretion of dietary triacylglycerol, as well as the size of the chylomicron particle (Weintraub et al. 1988).

The fatty acid composition of a chylomicron particle has been shown to be an important determinant of its metabolism in the circulation. Unsaturated fatty acids, especially PUFAs, were shown to increase the size of chylomicron particles compared with those from a test meal containing SFAs. After their release into the circulation, large triacylglycerol-rich chylomicrons are preferentially hydrolyzed by lipoprotein lipase, and removal of 70–90% of the triacylglycerol produces a cholesterol ester–rich particle termed a chylomicron remnant. Before their uptake by a receptor-mediated process in the liver, the particles interact with hepatic lipase, which further hydrolyzes triacylglycerol and phospholipid .

Van Greevenbroek et al. showed that unsaturated fatty acids, especially oleic acid, to be more efficiently incorporated into lipoproteins by the microsomal triacylglycerol transfer protein (MTP). It had been thought that MTP was involved only in the co-translational lipidation of apo B, protecting the protein from early intracellular degradation. However, MTP was shown in Caco-2 cells to be involved in the further lipidation of the primordial particle along the secretory pathway (van Greevenbroek et al. 1996). These data suggest that the short-term ingestion of olive oil may increase the number of apo B-48–containing particles by modulating the activity or expression of MTP or the messenger RNA editing enzyme within the enterocyte. Jackson et al demonstrated that the short-term ingestion of olive oil increases the number of triacylglyerol-rich chylomicron particles, compared with other oils. (Jackson, et al, 2002)

Along with these results, aIso in this study the short-term ingestion of olive oil induced a significant elevation of triglyceride levels, after only three hours from the gavage, in the serum of mice where the clearance of chylomicron remnants was inhibited, suggesting that olive oil may influence one or several steps involved in the formation or metabolism of the nascent chylomicrons in the circulation.

Other studies examined the effect of CLA on triglycerides, for instance, Lee et al showed that CLA significantly reduced the plasmatic concentration of triacylglycerols and LDL-cholesterol. (Lee et al. 1994)

In humans, few studies evaluated the result of CLA supplementation in the metabolism of plasmatic lipoproteins. In a placebo-controlled study, Noone et al. supplemented normolipidemic individuals with 3 g of CLA or placebo during eight weeks. At the end of study, they verified that the supplement of CLA led to a significant reduction of the concentration of VLDL cholesterol and plasmatic triacylglycerols without altering the content of HDL-cholesterol (Noone et al. 2002).

The results obtained in this study demonstrated that triglyceride levels can be modulated differently, after a short term ingestion of different dietary fatty acids. In agreement with these reports, in our studies higher serum triglyceride levels were observed in mice given a bolus dose of either olive oil, or sunflower oil (Figure 23). In contrast, serum triglyceride levels did not change, after a short term administration of either CLA t10,c12 or CLA c9,t11 isomer (Figure 1).

Despite the number of studies in animal models and in vitro, whether dietary CLA could promote health in humans is still controversial. Caution should be used in recommending CLA to prevent diseases in humans until these health effects are fully proven and the mechanisms of action entirely understood.

Data from literature strongly indicate that dietary CLA can perturb Vitamin A metabolism in rodent models ((Banni et al. 1999; Carta et al. 2002) and potentially also in humans *(data non published, See table 1).* Given this interference and given the fact that CLA controls many biological functions, has become crucial to understand the molecular mechanisms of this interaction, as we cannot rule out that this changes in vitamin A metabolism could be responsible for both beneficial and/or detrimental effects of dietary CLA.

Investigating the mechanisms underlying the effects of CLA on retinoid metabolism will help us advance our current understanding of some aspects of the vitamin A metabolism such as the mechanism regulating the secretion of hepatic ROH or those regulating dietary vitamin A absorption, that are still not fully clarified.

Metabolic studies have revealed that most of the retinyl esters in plasma are present in small chylomicrons; significant amounts are found in large chylomicrons and smaller amounts are found in very-low-density lipoprotein (VLDL) (Lemieux et al. 1998). Studies have been carried out in differentiated Caco-2 cells under postprandial conditions to determine the mechanism of retinol secretion by the intestine. In these studies, investigators found that a significant amount of retinyl ester was secreted mainly with chylomicrons independent of the rate of retinol uptake and intracellular levels of free or esterified retinol (Nayak et al. 2001).Pluronic L81, which inhibits the secretion of chylomicrons, decreased the secretion of retinyl ester and did not result in their increased secretion with smaller lipoproteins. This suggests that intestinal retinyl ester secretion is a highly specific and regulated process that is dependent on the assembly and secretion of chylomicrons. A significant amount of retinol is also secreted into the portal circulation, probably as free retinol; this is expected to be physiologically significant in pathological conditions that affect the secretion of chylomicrons (Hollander 1980). However, very little is known about the regulation of the retinol secretion by this pathway.

What is the origin of vitamin A that accumulates in the liver upon CLA feeding?

Effect of an acute dietary CLA administration on vitamin A intestinal absorption

The published study from Ortiz et al. raised the question about the origin of vitamin A that accumulates in storage tissues upon CLA feeding. We examined the hypothesis that dietary CLA may increase the intestinal absorption of retinoids, by altering assembly and secretion of retinyl ester-containing chylomicrons and resulting in its specific accumulation in liver.

To start gaining insights on the effect of CLA intake on intestinal absorption of vitamin A we took advantage of the pharmacological inhibitor of hydrolysis and clearance of chylomicrons, P-407. Administration of P-407 to the mice by IP injection optimizes the chance of detecting vitamin A of intestinal origin in the serum.

The analysis of [^{3}H] retinoids in the liver of these mice showed the efficacy of P-407 in reducing RE-containing chylomicrons clearance by the liver (Figure 11). Reverse phase HPLC followed by fractions collection revealed that when the chylomicrons clearance is inhibited, a single dose of either CLA isomer increases the levels of [^{3}H] retinoids circulating in chylomicron particles. In addition, total [^{3}H] counts/min ROH + RE fractions were reduced in the small intestine of mice gavaged with olive oil + either CLA isomer (Figure 10). We interpret these results as an effect of dietary CLA in stimulating the intracellular processing of vitamin A in the enterocytes thus reducing [^{3}H] retinoid levels in the small intestine and increasing those in the bloodstream. LRAT mRNA expression levels in the small intestine showed a trend towards an upregulation when mice were given a oral bolus of either CLA isomer. We suppose that due to high standard deviation in the control group, this increase did not reach statistically significance. Despite this limitation, these results suggest that the intake of both CLA isomers may cause a rapid esterification of ROH into RE. We also analyzed the expression levels of MTTP, a key enzyme involved in the assembly of the chylomicron in the small intestine and no significant difference was observed in mRNA levels of this enzyme (Figure 14)

How does vitamin A metabolism change in response to a single acute CLA administration?

We also aimed at investigating the effects of a single acute administration of dietary CLA on RBP mediated hepatic retinol secretion and its redistribution towards peripheral tissues. In the case of wild-type mice gavaged with the same mixture of ROH and CLA described above, without the inhibition of hydrolysis and clearance of chylomicrons, not only hepatic (Figure 15), but also total serum [^{3}H] retinoids and specifically [^{3}H]ROH levels were elevated (Figure 16 A-B), suggesting that acute administration of CLA enhances hepatic ROH uptake and likely its rapid resecretion into the blood stream bound to its sole specific transport protein, retinol-binding protein (RBP). In contrast, western blot analysis of the levels of RBP in the serum of these mice showed no statistically significant differences with any of the CLA isomer (data not shown). We think that this is due to the fact that steady state levels of retinol in the serum were not perturbed by a single dose of CLA. Thus, only with the use of a sensitive technique such as radiolabeled vitamin A measurement, we were able to follow the distribution of recently ingested vitamin A and thus notice these alterations.

We next tried to confirm our hypothesis by using mice lacking RBP. In fact, in the absence of RBP, no statistically significant changes were observed in serum retinoids [^{3}H] levels, regardless of the type of CLA isomer supplemented with the gavage, demonstrating for the first time that, unlike the chronic feeding (Ortiz et al, 2009), both CLA isomers induce an increased secretion of the complex ROH-RBP from the liver and its rapid resecretion into the circulation, when acutely administered. In addition, the elevated total [^{3}H] retinoid levels in adipose tissue, an important retinoid storage site, and in the small intestine, upon either CLA isomer administration in the absence of P-407, suggest that RBP mediates a rapid redistribution of recently ingested vitamin A to the periphery. Realtime RT-PCR analysis showed that hepatic mRNA levels of RBP were not affected by the acute CLA administration. (Figure 19). These data are consistent with the hypothesis that CLA t10,c12 and CLA c9,t11 stimulate secretion of the complex retinol-RBP from the liver through mechanisms that do not involve transcriptional events (Ortiz et al. 2009). In the liver of mice gavaged with CLA c9,t11 LRAT mRNA levels showed a trend towards an upregulation compared to mice gavaged with olive oil, suggesting that this isomer might induce a response of the liver toward a rapid formation of retinyl ester. To gain some insights into the mechanism that leads to increased

hepatic uptake of recently ingested retinoid in the liver, after an acute CLA administration, we measured the expression levels of LRP and LDLr, receptors involved in the uptake of chylomicron remnants (Cooper 1997). Surprisingly, we observed a downregulation of LRP caused by either CLA isomer and decreased levels of LDLr induced only by CLA c9,t11 (Figure 18). Data from literature showed that there is a significant LDLR-independent clearance, accounting for as much as 25%-50% of the hepatic remnant uptake (Ishibashi et al, 1996) and suggesting that an additional mechanism exists in the liver. It has also been shown that cell-surface heparin sulfate proteoglycans (HSPGs) are critically important in the clearance of remnants lipoproteins, independent of LDLr family members (Mahley et al. 2007).We can hypothesize that one of these mechanisms might be responsible for the increased retinoid counts in the liver upon CLA gavage, but further experiments are required to elucidate this aspect.

Comparing the changes in vitamin A metabolism in response to an acute *versus* a chronic CLA administration

We examined the effects of a purified diet supplemented with 1% CLA *t10,c12* or 1%CLA *c9,t11* on circulating levels of ROH and RBP. We were able to confirm previously published data by Ortiz et al. who demonstrated that CLA t10,c12 is the only isomer that enhanced serum levels of ROH and its specific carrier, RBP, upon four weeks of dietary CLA supplementation (Figure 20).

As a next step we analyzed the steady state levels of retinol and RBP protein levels in the serum of mice chronically fed with the purified supplemented diets and then subjected to gavage administration. Surprisingly, we noticed that the acute intake of either CLA isomer blunted the differential effects of each isomer on serum ROH and RBP protein levels, observed in mice chronically fed with CLA (Table 4 A-B). Furthermore, we measured the kinetic of recently ingested vitamin A and we noticed a statistically significant reduction in serum total [^{3}H] retinoid levels (ROH +RE fractions), specifically in the ROH fraction. It's also important to highlight that [^{3}H]RE levels did not change among groups. We hypothesize that this results might be due to an impairment of the absorption of vitamin A and its incorporation into the chilomycrons as RE, in a condition where the metabolism was already stimulated for a long time by either CLA isomer. Further investigations are needed to unequivocally prove this hypothesis.

To better understand how the retinoid pool flux between tissues is regulated, we quantified the effect of dietary CLA *t10,c12* and CLA *c9,t11* on liver stores. Interestingly, we observed that steady state RE levels significantly increased in mice given different types of gavage mixtures, compared to those that received only the chronic feeding (Table 5 A-B). These results raised the question about the origin of these higher RE levels. We hypothesized that this difference might be due to a different rate of retinol oxidation and/or esterification, probably depending on the metabolic status of this tissue. In both sets of experiments (chronic diet and chronic diet + gavage) the steady state levels analysis showed that CLA *t10,c12* is the only isomer that decreased significantly hepatic RE concentrations, thus supporting previous data (Ortiz et al) that demonstrated the ability of this isomer to stimulate the secretion of the complex ROH-RBP from the liver. When we analyzed the effect of a single dietary CLA intake on the rapid turnover of radiolabeled vitamin A in the liver, we

noticed the same trend as the steady state level, specifically [^{3}H]RE were significantly decreased upon CLA *t10,12* gavage administration (Figure 22). Finally, we examined the kinetic of distribution of radiolabeled vitamin A in another important retinoid storage tissue and we observed a significant reduction in total [^{3}H] retinoid levels upon CLA *t10,12* gavage.

Taken together these results suggest that an acute administration of either CLA *t10,12 and* CLA *c9,t11* given to mice already chronically stimulated with a diet supplemented with the same CLA isomer, probably induced a reduction in the absorption of recently ingested vitamin A from the intestine, thus resulting in lower levels of [^{3}H] retinoid in the liver and in the adipose tissue. This hypothesis needs to be proven with further experiments.

Differential effects of Sunflower oil, Flaxseed oil and Olive oil on vitamin A absorption

Finally, we investigated how vitamin A metabolism changes in response to an acute administration of oils that differ in the fatty acid content and whether these oils might alter vitamin A incorporation into the chylomicrons.

The mechanisms of digestion and absorption of dietary vitamin A are currently a matter of debate, and they have not been elucidated completely yet.

It's known that vitamin A, carotenoids, and polyunsaturated fatty acids (PUFA) are closely related nutritionally (Tomassi et al. 1983). For example, diets rich in PUFA, lower the efficiency of utilization of dietary vitamin A and carotenoids by enhancing their peroxidation in the gastro-intestinal tract (Auger et al. 1942).

To start gaining insights on the effect of an acute administration of olive oil, sunflower oil and flaxseed oil on intestinal absorption of vitamin A, we inhibited the hydrolysis and clearance of chylomicrons, by injecting the mice with P-407, thus optimizing the chance of detecting vitamin A of intestinal origin in the serum.

We observed that an acute administration of these oils affected both steady state and [^{3}H] retinoid levels in the serum of these mice, together with total [^{3}H] retinoid content in the small intestine. Specifically, when we followed the turnover of recently ingested radiolabeled vitamin A, we observed that sunflower oil and flaxseed oil compared to olive oil induced a marked reduction of [^{3}H] ROH in both serum and small intestine, along with decreased [^{3}H] RE in the small intestine. In brief, different oils with different fatty acid content might affect the vitamin A status of the body within a short time from the ingestion.

These oils are characterized by the high content of oleic acid (about 70% in olive oil), linoleic acid (about 50% in sunflower oil) and alpha-linolenic acid (about 50% in linseed oil), demonstrating that the number of double bonds and/or their positional isomers, as the case of CLA, influence vitamin A absorption.

Vitamin A deficiency is estimated to affect 140 million preschool-aged children and more than 7 million pregnant women. It contributes to the deaths of over one million children each year (Li et al. 2003). In addition, a subclinical vitamin A deficiency may emerge in particular physio-pathological conditions due to a higher requirement and/or higher degradation, or impaired absorption. Our data, shed some lights on a still unravel issue, i.e. how dietary fats influence

vitamin A absorption. It is also relevant the “nutrient-nutrient” interactions aiming at the identification of those interactions that might alter absorption and distribution of vitamin A.

This is an important issue because vitamin A or its precursor beta-carotene are normally present in fatty foods which according to their fatty acid composition may affect vitamin A absorption and tissue distribution. Furthermore, in case of fat malabsorption either due to a pathological conditions or consequent to pharmacological treatments such as lipase inhibitors vitamin A absorption may be dramatically impaired.

Our data lay the foundation for new studies aimed at assessing those nutritional factors that influence vitamin A absorption and tissue distribution and develop guidelines for optimal dietary intervention in vitamin A deficiency.

Conclusions and future perspectives

The results obtained in this study provide new interesting evidences about the interaction between important macronutrients, like lipids, needed for energy storage, structural composition of cell membranes and as signaling molecules, and micronutrients, like vitamin A, required by living organisms throughout the lifecycle. A special interest was placed on Conjugated Linoleic Acid (CLA), since numerous reports demonstrated its multiple biological activities and indicated its ability to perturb vitamin A metabolism, not only in rodent models, but potentially also in humans. Unlike the chronic feeding, where the two most biological active isomers, CLA *t10,c12* and *cis 9,trans 11* had distinct and common effects on vitamin A metabolism, in influencing hepatic retinol storage, secretion and delivery to the periphery of the body. This study showed for the first time that, this two isomer when acutely administered are able together to enhance retinoids uptake from the liver, a rapid resecretion of the ROH-RBP complex from the liver, into the bloodstream and finally its redistribution through the periphery. To further elucidate these actions, in future we propose to investigate whether the rate of RBP secretion from liver parenchimal cells is affected by CLA feeding, by using mouse models in which formation of vitamin A stores (LRAT -/-) and/or mobilization of hepatic retinol is impared (RBP -/-). Moreover, our results give also a strong suggestion of altered intestinal absorption of vitamin A, when either CLA isomer is acutely administered. It would be interesting to perform additional realtime RT-PCR analysis on the small intestine of the various groups of mice to assess the expression levels of other key players of intestinal RE-containing chylomicrons assembly and secretion, such as apoA-IV, a protein synthesized in the enterocytes and associated with nascent chylomicrons, whose expression increases upon fat feeding. To further investigate on the effect of a short-term CLA administration on vitamin A metabolism, it would be also important to analyze apoB 48 protein levels in the serum of these mice, since apoB 48 is a unique protein, exclusively sinthesized by the small intestine and associated to chylomicrons remnants. In the long term, the result of this study could help the scientific community to establish dietary guidelines for intake and tolerable upper limits of conjugated linoleic acid.

In addition, this study provides also some information about the effects of different types of oils on vitamin A metabolism, upon acute feeding. These results suggest that the oils used in the experiments: olive oil, sunflower oil and flaxseed oil are able to modulate retinoids levels

in the circulation and in the small intestine at different extends, probably due to a fatty acid-specific effect.

In this case it would be interesting investigating the effects on cellular retinol-binding protein type II (CRBP II): a key enzyme localized in small intestinal epithelial cells and that plays a important roles in intestinal absorption. Previously published data found that jejunal CRBP II mRNA and its protein levels in rats fed a high-fat (corn oil) dietwere more than twofold greater than in rats fed a low-fat diet (Goda, Yasutake et al. 1994). Unsaturated fatty acids, e.g., oleic acid,linoleic acid, and α-linolenic acid, enhanced CRBP II mRNA levels, whereas medium-chain fatty acids and

saturated fatty acids had little effect on CRBP II mRNA levels (24).

Many studies in literature investigated the long term effect of different dietary fatty acids on lipoprotein metabolism, but very few focused on the short term effect especially on the absorption and utilization of vitamin A.

For instance, a study from Harris W.S. et al showed that a gavage with emulsified soybean oil reduced the postprandial chylomicron triacylglycerol levels in rats fed with a diet rich in n-3 PUFA for 2 weeks, suggesting that the n-3 fatty acids feeding reduced chylomicron production/secretion from the intestine. They also noticed reduced plasma and hepatic levels of labeled retinyl esters in the treated animals, suggesting that peripheral removal of chylomicron-borne [^{3}H] RE was accelerated.

Given these interferences, many questions raise from this study and more mechanisms of action still need to be fully elucidated. Thus, it is very important to further investigate the interactions between different types of nutrient, with a special attention to the duration and type of the dietary treatment and the metabolic status of the body, in order to develop rationally-based dietary recommendations to ensure human health.

Auger, T. and J. Pediat (1942). "Abstracts from Current Literppature." Archives of Pediatrics and Adolescent Medicine **64**(2): 356.

Banni, S., E. Angioni, V. Casu, M. Melis, S. Scrugli, G. Carta, F. Corongiu and C. Ip (1999). "An increase in vitamin A status by the feeding of conjugated linoleic acid." Nutrition and cancer **33**(1): 53-57.

Batten, M. L., Y. Imanishi, et al. (2004). "Lecithin-retinol acyltransferase is essential for accumulation of all-trans-retinyl esters in the eye and in the liver." J Biol Chem **279**(11): 10422-10432.

Biesalski, H. K., J. Frank, et al. (1999). "Biochemical but not clinical vitamin A deficiency results from mutations in the gene for retinol-binding protein." Am J Clin Nutr **69**: 931-936.

Blaner, W. S. and J. A. Olson (1994). Retinol and retinoic acid metabolism. The Retinoids, Biology, Chemistry and Medicine. M. B. Sporn, A. B. Roberts and D. S. Goodman. New York, NY, Raven Press**:** 229-256.

Carta, G., E. Angioni, E. Murru, M. Melis, S. Spada and S. Banni (2002). "Modulation of lipid metabolism and vitamin A by conjugated linoleic acid." Prostaglandins, Leukotrienes and Essential Fatty Acids **67**(2-3): 187-191.

Cooper, A. D. (1997). "Hepatic uptake of chylomicron remnants." J Lipid Res **38**(11): 2173-2192.

Goldberg, I. J. (1996). "Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis." J Lipid Res **37**(4): 693-707.

Goodman, D. S., H. S. Huang, et al. (1965). "Tissue distribution of newly absorbed vitamin A in the rat." J Lipid Res **6**: 390-396.

Gronowska-Senger, A. and A. Rupniewska (1979). "Intake of essential unsaturated fatty acids and vitamin E, and the demand for vitamin A." Bromatologia i Chemia Toksykologiczna **12**: 13–19.

Harrison, E. H. (2005). "Mechanisms of digestion and absorption of dietary vitamin A." Annu Rev Nutr **25**: 87-103.

Hofmann, A. and B. Borgström (1963). "Hydrolysis of long-chain monoglycerides in micellar solution by pancreatic lipase." Biochimica et Biophysica Acta **70**: 317-331.

Hollander, D. (1980). "Retinol lymphatic and portal transport: influence of pH, bile, and fatty acids." American Journal of Physiology- Gastrointestinal and Liver Physiology **239**(3): G210.

Hussain, M. (2000). "A proposed model for the assembly of chylomicrons." Atherosclerosis **148**(1): 1-15.

Kawaguchi, R., J. Yu, et al. (2007). "A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A." Science **315**(5813): 820-825.

Lemieux, S., R. Fontani, K. Uffelman, G. Lewis and G. Steiner (1998). "Apolipoprotein B-48 and retinyl palmitate are not equivalent markers of postprandial intestinal lipoproteins." Journal of lipid research **39**(10): 1964.

Li, E. and P. Tso (2003). "Vitamin A uptake from foods." Current Opinion Lipidology **14**: 241-247.

Liu, L. and L. J. Gudas (2005). "Disruption of the lecithin:retinol acyltransferase gene makes mice more susceptible to vitamin A deficiency." J Biol Chem **280**(48): 40226-40234.

Lombardo, D. and O. Guy (1980). "Studies on the substrate specificity of a carboxyl ester hydrolase from human pancreatic juice. II. Action on cholesterol esters and lipid-soluble vitamin esters." Biochimica et Biophysica Acta (BBA)-Enzymology **611**(1): 147-155.

Mahley, R. and Y. Huang (2007). "Atherogenic remnant lipoproteins: role for proteoglycans in trapping, transferring, and internalizing." Journal of Clinical Investigation **117**(1): 94-98.

Monaco, H. L., M. Rizzi, et al. (1995). "Structure of a complex of two plasma proteins: transthyretin and retinol-binding protein." Science **268**(5213): 1039-1041.

Nayak, N., E. Harrison and M. Hussain (2001). "Retinyl ester secretion by intestinal cells: a specific and regulated process dependent on assembly and secretion of chylomicrons." Journal of lipid research **42**(2): 272.

O'Byrne, S. M., N. Wongsiriroj, et al. (2005). "Retinoid absorption and storage is impaired in mice lacking lecithin:retinol acyltransferase (LRAT)." J Biol Chem **280**(42): 35647-35657.

Olivecrona, T. and G. Bengtsson-Olivecrona (1993). "LIpoprotein lipase and hepatic lipase." Current Opinion in Lipidology **4**: 187-196.

Ortiz, B., L. Wassef, et al. (2009). "Hepatic retinol secretion and storage are altered by dietary conjugated linoleic acid (CLA): common and distinct actions of CLA c9, t11 and t10, c12 isomers." The Journal of Lipid Research: M900054.

Quadro, L., W. S. Blaner, et al. (1999). "Impaired retinal function and vitamin A availability in mice lacking retinol-binding protein." Embo J **17**: 4633-4644.

Quadro, L., L. Hamberger, et al. (2003). "Understanding the Physiological Role of Retinol-Binding Protein in Vitamin A Metabolism Using Transgenic and Knockout Mouse Models." Mol Aspect Med **24**: 421-430.

Ribaya-Mercado, J. (2002). "Influence of Dietary Fat on-Carotene Absorption and Bioconversion into Vitamin A." Nutr. Rev **60**: 104-110.

Rowe, L. and E. Wills (1976). "The effect of dietary lipids and vitamin E on lipid peroxide formation, cytochrome P-450 and oxidative demethylation in the endoplasmic reticulum." Biochemical Pharmacology **25**(2): 175-179.

Astrup, A., J. Dyerberg, P. Elwood, K. Hermansen, F. B Hu, M. Uhre Jakobsen, F. J Kok, R. M Krauss, J. M. Lecerf, P. LeGrand, P. Nestel, U. Rise´rus, T. Sanders, A. Sinclair, S. Stender, T. Tholstrup and W. Willett (2011). "The role of reducing intakes of saturated fat in the prevention of cardiovascular disease: where does the evidence stand in 2010?" American Journal of Clinical Nutrition.

Auger, T. and J. Pediat (1942). "Abstracts from Current Literppature." Archives of Pediatrics and Adolescent Medicine **64**(2): 356.

Banni, S. (2002). "Conjugated linoleic acid metabolism." Current Opinion Lipidology **13**: 261-266.

Banni, S., E. Angioni, V. Casu, M. Melis, S. Scrugli, G. Carta, F. Corongiu and C. Ip (1999). "An increase in vitamin A status by the feeding of conjugated linoleic acid." Nutrition and cancer **33**(1): 53-57.

Banni, S., A. Petroni, M. Blasevich, G. Carta, L. Cordeddu, E. Murru, M. P. Melis, A. Mahon and M. A. Belury (2004). "Conjugated linoleic acids (CLA) as precursors of a distinct family of PUFA." Lipids **39**: 1143-1146.

Batten, M. L., Y. Imanishi, T. Maeda, D. C. Tu, A. R. Moise, D. Bronson, D. Possin, R. N. Van Gelder, W. Baehr and K. Palczewski (2004). "Lecithin-retinol acyltransferase is essential for accumulation of all-trans-retinyl esters in the eye and in the liver." J Biol Chem **279**(11): 10422-10432.

Benjamin, S. and F. Spener (2009). "Conjugated linoleic acids as functional food: an insight into their health benefits." Nutrition & metabolism **6**(1): 36.

Bhattacharya, A., J. Banu, M. Rahman, J. Causey and G. Fernandes (2006). "Biological effects of conjugated linoleic acids in health and disease." Journal of Nutritional Biochemistry **17**: 789-810.

Biesalski, H. K., J. Frank, S. C. Beck, F. Heinrich, B. Illek, R. Reifen, H. Gollnick, M. W. Seeliger, B. Wissinger and Z. E. (1999). "Biochemical but not clinical vitamin A deficiency results from mutations in the gene for retinol-binding protein." Am J Clin Nutr **69**: 931-936.

Blaner, W. S. and J. A. Olson (1994). Retinol and retinoic acid metabolism. The Retinoids, Biology, Chemistry and Medicine. M. B. Sporn, A. B. Roberts and D. S. Goodman. New York, NY, Raven Press**:** 229-256.

Blomhoff, R. (1994). Vitamin A in health and disease, CRC.

Carta, G., E. Angioni, E. Murru, M. Melis, S. Spada and S. Banni (2002). "Modulation of lipid metabolism and vitamin A by conjugated linoleic acid." Prostaglandins, Leukotrienes and Essential Fatty Acids **67**(2-3): 187-191.

Churruca, I., A. Fernández-Quintela and M. P. Portillo (2009). "Conjugated linoleic acid isomers: differences in metabolism and biological effects." Biofactors **35**(1): 105-111.

Clément, L., H. Poirier, I. Niot, V. Bocher, M. Guerre-Millo, S. Krief, B. Staels and P. Besnard (2002). "Dietary trans-10, cis-12 conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse." Journal of lipid research **43**(9): 1400.

Cooper, A. D. (1997). "Hepatic uptake of chylomicron remnants." J Lipid Res **38**(11): 2173-2192.

Fritsche, J. R. R. and H. Steinhart (1999). "Formation, contents, and estimation of daily intake of conjugated linoleic acid isomers and trans-fatty acids in foods." Advances in Conjugated Linoleic Acid Research **1**: 378-396.

Goldberg, I. J. (1996). "Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis." J Lipid Res **37**(4): 693-707.

Goodman, D. S., H. S. Huang and T. Shiratori (1965). "Tissue distribution of newly absorbed vitamin A in the rat." J Lipid Res **6**: 390-396.

Gronowska-Senger, A. and A. Rupniewska (1979). "Intake of essential unsaturated fatty acids and vitamin E, and the demand for vitamin A." Bromatologia i Chemia Toksykologiczna **12**: 13–19.

Harrison, E. H. (2005). "Mechanisms of digestion and absorption of dietary vitamin A." Annu Rev Nutr **25**: 87-103.

Hofmann, A. and B. Borgström (1963). "Hydrolysis of long-chain monoglycerides in micellar solution by pancreatic lipase." Biochimica et Biophysica Acta **70**: 317-331.

Hollander, D. (1980). "Retinol lymphatic and portal transport: influence of pH, bile, and fatty acids." American Journal of Physiology- Gastrointestinal and Liver Physiology **239**(3): G210.

Hussain, M. (2000). "A proposed model for the assembly of chylomicrons." Atherosclerosis **148**(1): 1-15.

Janke, J., S. Engeli, M. Boschmann, F. Adams, J. Böhnke, F. Luft, A. Sharma and J. Jordan (2006). "Retinol-binding protein 4 in human obesity." Diabetes **55**(10): 2805.

Kawaguchi, R., J. Yu, J. Honda, J. Hu, J. Whitelegge, P. Ping, P. Wiita, D. Bok and H. Sun (2007). "A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A." Science **315**(5813): 820-825.

Kelly, G. (2001). "Conjugated linoleic acid: a review." Alternative medicine review: a journal of clinical therapeutic **6**(4): 367.

Kennedy, A., K. Martinez, S. Schmidt, S. Mandrup, K. LaPoint and M. McIntosh (2010). "Antiobesity mechanisms of action of conjugated linoleic acid." Journal of Nutritional Biochemistry **21**: 171-179.

Lemieux, S., R. Fontani, K. Uffelman, G. Lewis and G. Steiner (1998). "Apolipoprotein B-48 and retinyl palmitate are not equivalent markers of postprandial intestinal lipoproteins." Journal of lipid research **39**(10): 1964.

Li, E. and P. Tso (2003). "Vitamin A uptake from foods." Current Opinion Lipidology **14**: 241-247.

Liu, L. and L. J. Gudas (2005). "Disruption of the lecithin:retinol acyltransferase gene makes mice more susceptible to vitamin A deficiency." J Biol Chem **280**(48): 40226-40234.

Lombardo, D. and O. Guy (1980). "Studies on the substrate specificity of a carboxyl ester hydrolase from human pancreatic juice. II. Action on cholesterol esters and lipid-soluble vitamin esters." Biochimica et Biophysica Acta (BBA)-Enzymology **611**(1): 147-155.

Mahley, R. and Y. Huang (2007). "Atherogenic remnant lipoproteins: role for proteoglycans in trapping, transferring, and internalizing." Journal of Clinical Investigation **117**(1): 94-98.

Martin, J. and K. Valeille (2002). "Conjugated linoleic acids: all the same or to everyone its own function?" Reproduction Nutrition Development **42**(6): 525-536.

Monaco, H. L., M. Rizzi and A. Coda (1995). "Structure of a complex of two plasma proteins: transthyretin and retinol-binding protein." Science **268**(5213): 1039-1041.

Nayak, N., E. Harrison and M. Hussain (2001). "Retinyl ester secretion by intestinal cells: a specific and regulated process dependent on assembly and secretion of chylomicrons." Journal of lipid research **42**(2): 272.

O'Byrne, S. M., N. Wongsiriroj, J. Libien, S. Vogel, I. J. Goldberg, W. Baehr, K. Palczewski and W. S. Blaner (2005). "Retinoid absorption and storage is impaired in mice lacking lecithin:retinol acyltransferase (LRAT)." J Biol Chem **280**(42): 35647-35657.

Olivecrona, T. and G. Bengtsson-Olivecrona (1993). "LIpoprotein lipase and hepatic lipase." Current Opinion in Lipidology **4**: 187-196.

Ortiz, B., L. Wassef, E. Shabrova, L. Cordeddu, S. Banni and L. Quadro (2009). "Hepatic retinol secretion and storage are altered by dietary conjugated linoleic acid (CLA): common and distinct actions of CLA c9, t11 and t10, c12 isomers." The Journal of Lipid Research: M900054.

Pariza, M. W. and W. A. Hargraves (1985). "A beef-derived mutagenesis modulator inhibits initiation of mouse epidermal tumors by 7,12-dimethylbenz[a]anthracene." Carcionogenesis **6**: 591-593.

Park, Y., K. J. Albright, W. Liu, J. M. Storkson, M. E. Cook and M. W. Pariza (1997). "Effect of conjugated linoleic acid on body composition in mice." Lipids **32**: 853-858.

Perez-Matute, P., A. Marti, J. Martinez, M. Fernandez-Otero, K. Stanhope, P. Havel and M. Moreno-Aliaga (2005). "Eicosapentaenoic fatty acid increases leptin secretion from primary cultured rat adipocytes: role of glucose metabolism." American Journal of Physiology-Regulatory, Integrative and Comparative Physiology **288**(6): R1682.

Quadro, L., W. S. Blaner, D. J. Salchow, S. Vogel, R. Piantedosi, P. Gouras, S. Freeman, M. P. Cosma, V. Colantuoni and M. E. Gottesman (1999). "Impaired retinal function and vitamin A availability in mice lacking retinol-binding protein." Embo J **17**: 4633-4644.

Quadro, L., L. Hamberger, V. Colantuoni, M. Gottesman and W. S. Blaner (2003). "Understanding the Physiological Role of Retinol-Binding Protein in Vitamin A Metabolism Using Transgenic and Knockout Mouse Models." Mol Aspect Med **24**: 421-430.

Ribaya-Mercado, J. (2002). "Influence of Dietary Fat on-Carotene Absorption and Bioconversion into Vitamin A." Nutr. Rev **60**: 104-110.

Ross, A. (2010). "Diet in vitamin A research." Methods in molecular biology (Clifton, NJ) **652**: 295.

Rowe, L. and E. Wills (1976). "The effect of dietary lipids and vitamin E on lipid peroxide formation, cytochrome P-450 and oxidative demethylation in the endoplasmic reticulum." Biochemical Pharmacology **25**(2): 175-179.

Sampath, H. and J. Ntambi (2005). "Polyunsaturated fatty acid regulation of genes of lipid metabolism." Nutrition **25**(1): 317.

Sebedio, J. L., P. Juaneda, G. Dobson, I. Ramilison, J. C. Martin, J. M. Chardigny and W. Christie (1997). "Metabolites of conjugated isomers of linoleic acid (CLA) in the rat." Biochimica et Biophysica Acta **1345**: 5-10.

Siri-Tarino, P., Q. Sun, F. Hu and R. Krauss (2010). "Meta-analysis of prospective cohort studies evaluating the association of saturated fat with cardiovascular disease." American Journal of Clinical Nutrition.

Soprano, D. R. and W. S. Blaner (1994). Plasma retinol-binding protein. The Retinoids, Biology, Chemistry and Medicine. M. B. Sporn, A. B. Roberts and D. S. Goodman. New York, NY, Raven Press**:** 257-282.

Tomassi, G. and J. Olson (1983). "Effect of dietary essential fatty acids on vitamin A utilization in the rat." Journal of Nutrition **113**(3): 697.

Vogel, S., M. V. Gamble and W. S. Blaner (1999). Biosynthesis, Absorption, Metabolism and Transport of Retinoids. in Handbook of experimental pharmacology. Retinoids. The biochemical and molecular basis of vitamin A and retinoid action. H. Nau and W. S. Blaner. Heidelberg, Germany, Springer Verlag Publishing. **139:** 31-95.

Weintraub, M., R. Zechner, A. Brown, S. Eisenberg and J. Breslow (1988). "Dietary polyunsaturated fats of the W-6 and W-3 series reduce postprandial lipoprotein levels.

Chronic and acute effects of fat saturation on postprandial lipoprotein metabolism." Journal of Clinical Investigation **82**(6): 1884.

Yang, Q., T. E. Graham, N. Mody, F. Preitner, O. D. Peroni, J. M. Zabolotny, K. Kotani, L. Quadro and B. B. Kahn (2005). "Serum retinol binding protein 4 contibutes to insulin resistance in obesity and type 2 diabetes." Nature **436**(7049): 356-362.

Acknowledgements

I would like to express my gratitude to my supervisor, Prof. Sebastiano Banni, whose expertise, understanding, and patience, added considerably to my graduate experience and for having offered me the opportunity of spending part of my graduate training at Rutgers University, NJ, and working on an exciting research project at the Food Science Department.
A very special thanks goes out to Dr. Loredana Quadro, for her motivation and encouragement. She provided me with direction, technical support and became a very special mentor. I truly appreciated the amount of time and energy that she invested in guiding me throughout this work.
I must also acknowledge all the members of Dr. Quadro's lab for exchanges of knowledge, skills, ideas and experiences, which helped enrich the experience and made it unforgettable.
I would also like to thank my family for the support they provided me through my entire life and especially during these three years.